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*Founded by Jacques Loeb*

EDITORS

W. J. CROZIER

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W. J. V. OSTERHOUT

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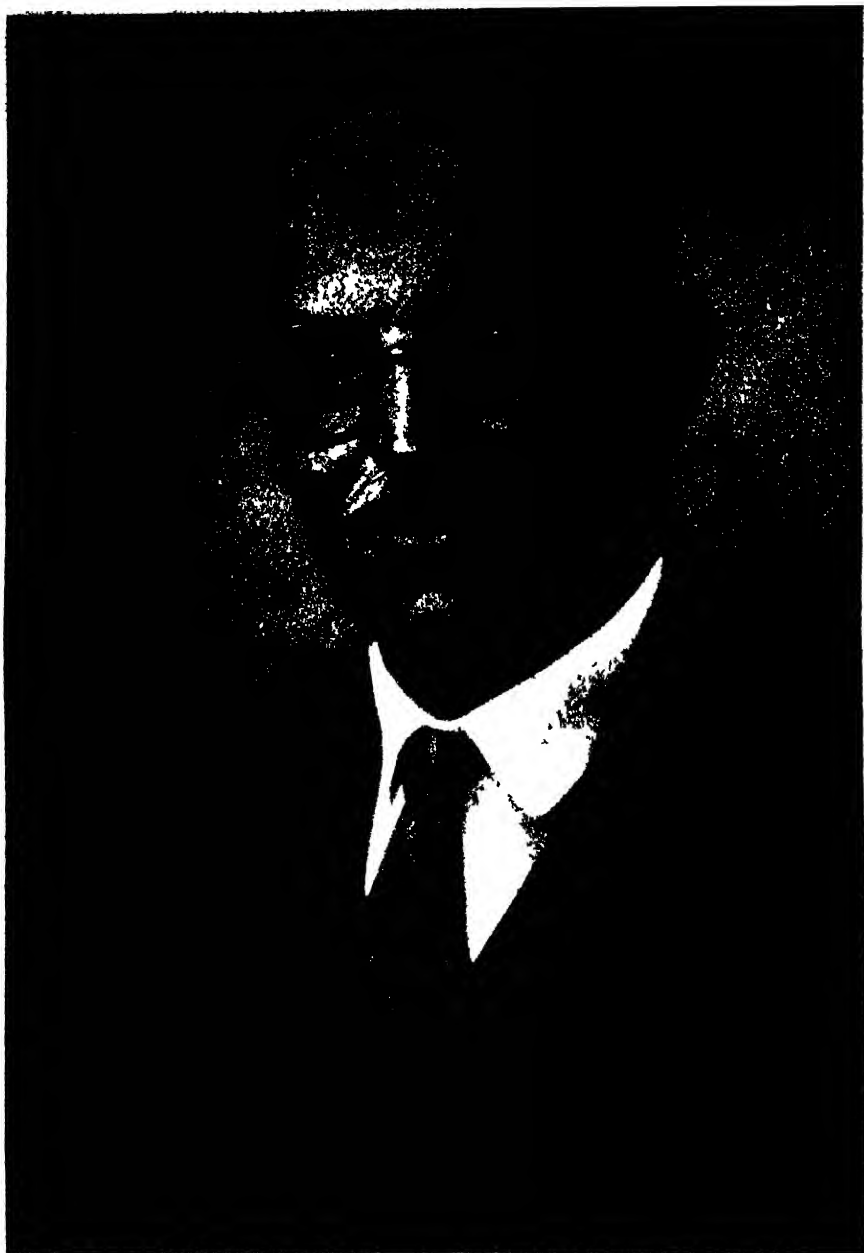
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*Photographed by Louis Schmidt*

*Yours very sincerely,  
H. J. V. Oettker*

Dedicated to

WINTHROP JOHN VANLEUVEN OSTERHOUT

*Member Emeritus of The Rockefeller Institute for Medical Research*

ON THE OCCASION OF HIS SEVENTIETH BIRTHDAY

AUGUST 2, 1941



# A PERFUSING SOLUTION FOR THE LOBSTER (*HOMARUS*) HEART AND THE EFFECTS OF ITS CONSTITUENT IONS ON THE HEART

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(Received for publication, April 4, 1941)

## INTRODUCTION

As indicated in the report on a perfusion solution for the crayfish heart (Cole, Helfer, and Wiersma, 1939), the inorganic composition of perfusing solutions for invertebrate hearts should agree closely with that of the serum of the animal used if normal behavior of the heart is to continue. Data have been accumulated on the serum of the lobster *Homarus americanus* (Cole and Kazalski, 1939; Parker and Cole, 1940; Cole, 1939-40 and 1941), which demonstrate that the concentrations of the common inorganic ions—sodium, potassium, calcium, magnesium, chloride and sulfate—vary differentially with the concentrations of those ions in the environment. Since many of the marine invertebrates studied in the laboratory are collected from shore and bay habitats, the sea water of which varies in inorganic composition from the average of open ocean water (Clarke, 1911; Thompson, 1936) parallel analyses of the environmental sea water should accompany those of the sera, if physiological solutions are to be prepared. To show clearly the differences, ratios of ionic concentrations in the serum to those in sea water, referred to total salt concentration of sea water (measured easily by chlorinity or millimoles of chloride per liter) may be used (Cole, 1939-40 and 1941).

The calcium content of lobster serum varies more than that of the other ions. It is believed that the variation may be correlated with the time since the last molt of the animal, as shown in the crab *Callinectes* by Hecht (1914). The value for calcium given in Table I is the average of nineteen individual sera analyzed during three summers (1938-40).

## RESULTS

A perfusing solution for the lobster heart made according to preliminary analysis of lobster serum, but with an increased total salt concentration to

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give isotonicity with serum (No. 2, Table I), was satisfactory for normal beating of the heart for as long as 26 hours.<sup>1</sup> Decreases and increases in the osmotic pressure of the solution up to 15 per cent (Nos. 2 *a* and 2 *b*) caused no significant changes in the rate or character of the heart beat for several hours. Environmental sea water and van't Hoff's solution, however, were both unsatisfactory for perfusion, since normal rate and amplitude of the heart beat continued only a few minutes, even though beating at decreased rate and amplitude might go on for 3 hours. Subsequent analyses of lobster serum, showing less potassium and calcium, resulted in the preparation of solution 35 containing less potassium and calcium, and more magnesium and sulfate, which caused decreased rate and amplitude of the beat. Solution 36, with the same amount of potassium and calcium as in No. 2, and the same amount of magnesium and sulfate as in No. 35, was satisfactory.

It should be noted that these solutions and the ones used later contained only the inorganic salts—sodium chloride, potassium chloride, calcium chloride, magnesium chloride, and magnesium sulfate. All organic and the minor inorganic constituents of the serum were absent. This may have been why the absolute amounts of calcium and potassium had to be increased above the amounts in serum. In other words, the missing constituents in inorganic perfusing solutions may be replaceable by increased amounts of potassium and calcium. On the other hand, the numbers of potassium and calcium ions per one hundred sodium ions in the best perfusing solutions (Nos. 2 and 36), while larger than the content of each ion in serum, show lower ratios to each other than in the serum. In the latter, the ratio of calcium to potassium per one hundred sodium ions is 2.0, while in the solutions it is 1.7.

Obviously, as for other hearts, critical ratios between the sodium, potassium, and calcium ions in a perfusing solution determine whether or not the lobster heart can continue normal beating. In order to determine those ratios and the specific effects of each ion, forty-two other solutions were tested. Some of the solutions and their effects are displayed in Table I.

Solutions of single components all caused arrest of the heart. Systolic arrest resulted from potassium chloride within 30 seconds, from urea within 5 minutes and from sodium chloride within 5 minutes (solutions 15, 52, and 1). Diastolic arrest resulted from magnesium sulfate within 6 seconds, from magnesium chloride within 15 seconds, from calcium chloride within 30 seconds, and from glucose within 50 seconds (solutions 51, 50, 26, and 53). None of these effects were irreversible, since recovery of the normal beat occurred within a few minutes, the order being just the reverse of the order of arrest.

<sup>1</sup> Details of the procedure used in preparing the animals and of testing perfusing fluids on the hearts *in situ* are fully described by Cole and Kazalski (1939) and by Cole, Helfer, and Wiersma, (1939). In the former report and in another by Cole and Parker (1940) preliminary results of perfusing the lobster heart are presented.

TABLE I

Compositions and effects of perfusing solutions on the lobster heart; pH = 7.3 to 7.5; temperature = 16.6 to 17.7°C.; rate of perfusion = 10 ml. per minute. Solutions efficiently buffered by adding 17.57 ml. of 0.5 M boric acid and 0.956 ml. of 0.5 M NaOH to each liter.

Solution	Millimoles per liter						No. of ions per 100 Na		Effects on heart
	Cl	Na	K	Ca	Mg	SO <sub>4</sub>	K	Ca	
Sea Water—Maine	500	458	8.5	11.4	52	32	1.86	2.49	Normal, 10 min.
Lobster serum	472	454	9.3	18.6	9.0	5.0	2.05	4.10	Not tested
Van't Hoff solution	554	454	10.0	10.0	35.0	17.0	2.20	2.20	Normal, 10 min.
No. 2	525	452	15.0	25.0	8.0	4.0	3.32	5.53	Normal, 26 hrs.
No. 2a	446	384	12.8	21.0	6.8	3.4	3.33	5.47	Normal, 20 hrs.
No. 2b	604	520	17.3	28.7	9.2	4.6	3.32	5.51	Normal, 19 hrs.
No. 36	533	456	15.0	25.0	12.0	6.0	3.29	5.48	Normal, 26 hrs.
No. 37	533	462	9.0	25.0	12.0	6.0	1.95	5.41	Normal, 20 hrs.
No. 38	528	461	15.0	20.0	12.0	6.0	3.25	4.34	Normal, 22 hrs.
No. 6	525	452	15.0	25.0	8.0	0	3.32	5.53	Normal, 3 hrs.
No. 5	525	460	15.0	25.0	0	0	3.26	5.44	Normal, 2 hrs.
No. 35	528	467	9.0	20.0	12.0	6.0	1.93	4.28	Dec. rate and ampl.
No. 1	525	525	0	0	0	0	0	0	Arrest in systole
No. 15	525	0	525	0	0	0	—	0	Arrest in systole
No. 26	526	0	0	263	0	0	0	—	Arrest in diastole
No. 50	526	0	0	0	263	0	0	0	Arrest in diastole
No. 51	0	0	0	0	525	525	0	0	Arrest in diastole
No. 52 urea 1 M	0	0	0	0	0	0	0	0	Arrest in systole
No. 53 glucose 1 M	0	0	0	0	0	0	0	0	Arrest in diastole
No. 8	500	484	16.0	0	0	0	3.30	0	Arrest in systole
No. 7	526	474	0	26.0	0	0	0	5.49	Arrest in systole
No. 21	504	496	0	0	4.0	0	0	0	Arrest in systole
No. 20	500	492	0	0	8.0	4.0	0	0	Arrest in systole
No. 9a	520	0	120	200	0	0	Ca/K = 1.67		Arrest in systole
No. 18a	530	470	0	26.0	8.0	4.0	0	5.53	Arrest in diastole
No. 16a	504	480	16.0	0	8.0	4.0	3.33	0	Arrest in systole
No. 41	533	467	4.0	25.0	12.0	6.0	0.86	5.35	Dec. rate and ampl.
No. 40	533	464	7.0	25.0	12.0	6.0	1.51	5.39	Dec. rate and ampl.
No. 46	538	451	15.0	30.0	12.0	6.0	3.32	6.65	Increased rate
No. 43	533	446	25.0	25.0	12.0	6.0	5.61	5.61	Dec. rate and ampl.
No. 47	533	441	30.0	25.0	12.0	6.0	6.80	5.67	Inc. tone and rate
No. 3	526	442	12.0	17.0	29.0	10.0	2.72	3.85	Dec. rate and ampl.
No. 45	523	466	15.0	15.0	12.0	6.0	3.22	3.22	Decreased rate
No. 44	518	471	15.0	10.0	12.0	6.0	3.18	2.12	Dec. rate and ampl.
No. 48	400	342	11.0	19.0	9.0	4.5	3.22	5.55	Inc. tone and rate
No. 49	666	570	19.0	31.0	15.0	7.5	3.33	5.44	Decreased rate
No. 29 LiCl	Li								
	525	452	15.0	25.0	8.0	4.0	3.32	5.53	Arrest in systole
No. 30 NaBr	Br								
	525	452	15.0	25.0	8.0	4.0	3.32	5.53	Dec. rate and ampl.
No. 31 NaI	I								
	525	452	15.0	25.0	8.0	4.0	3.32	5.53	Arrest in diastole

Among the binary solutions of salts, considerable differences were found. Potassium chloride and calcium chloride (No. 9 *a*) caused arrest in systole in less than 1 minute, indicating the predominating effect of potassium. Solutions of potassium chloride and magnesium chloride or magnesium sulfate, and of calcium chloride and magnesium chloride or magnesium sulfate were similarly effective in causing arrest, the former group in systole and the latter in diastole. Sodium chloride and magnesium chloride or magnesium sulfate (Nos. 20 and 21) were slightly better than sodium chloride alone, causing arrest in systole within 5 minutes. Sodium chloride and potassium chloride (No. 8) caused arrest in systole within 10 minutes, while sodium chloride and calcium chloride (No. 7) allowed normal beating for 30 minutes. The order of antagonism of sodium was therefore: calcium > potassium > magnesium. Calcium and magnesium did not antagonize potassium in the absence of sodium.

Tertiary and quaternary salt solutions were better than binary solutions provided they contained sodium, potassium, and calcium in certain proportions. Omission of potassium or sodium caused arrest in diastole (solution 18 *a*); omission of calcium caused arrest in systole (solution 16 *a*); but omission of magnesium or sulfate or both made no difference for as long as 3 hours.

The critical ratios between the sodium, potassium, and calcium ions were determined by a series of solutions in which the number of potassium and calcium ions per one hundred sodium ions were varied (solutions 3, 37, 38, 40, 41, 43-47). The results indicated that at least two potassium ions per one hundred sodium ions were necessary for normal behavior of the heart, provided there were five calcium ions present; three potassium and five calcium ions could be present, but three potassium ions were necessary if only four calcium ions were present. Within those narrow limits calcium could replace potassium and *vice versa*, without affecting the heart beat. If calcium or potassium were increased or decreased beyond those limits (solutions 35, 40, 46, and 47), the character of the heart beat was altered. For the crayfish (Cole, Helfer and Wiersma, 1939) the limits of the number of potassium and calcium ions per one hundred sodium ions were found to be from 1.8 and 6.8 to 2.6 and 6.6 respectively, showing narrower limits for calcium than in the lobster.

Decreasing or increasing the osmotic pressure of the perfusing solution by 25 per cent, without changing the ionic ratios of sodium, potassium, and calcium, caused increased tone and rate or decreased rate respectively (solutions 48 and 49). Adaptation to the former soon occurred and beating continued for several hours, but there was no adaptation to the latter, which caused irreversible diastolic arrest.

Solutions in which lithium replaced sodium, and bromide or iodide replaced chloride were all unsatisfactory, but in different degrees (solutions 29-31). Iodide caused quick arrest in diastole, lithium caused increased rate and tone

followed by arrest in systole within 6 minutes, while bromide allowed normal beating for about 12 minutes, followed by decreased rate and amplitude, and by arrest in diastole within 30 minutes. Lithium chloride behaves like sodium and potassium chlorides in causing systolic arrest. The bromide and iodide ions, however, show a marked difference from the chloride ion, since their sodium salts cause diastolic instead of systolic arrest.

In the earlier experiments the pH of the solutions was adjusted to that of the serum ( $7.4 \pm 0.1$ ) by adding small amounts of 1.0 N sodium hydroxide. Varying the pH from 7.0 to 8.0 caused no significant changes in frequency, tone, or amplitude of the heart beat. At lower or higher values, increases in tone and rate accompanied by decreases in amplitude occurred. The buffering capacity of such solutions was insufficient to maintain a pH of 7.4 in the pericardial chamber for more than about 4 hours of perfusion. In later experiments, the solutions were more efficiently buffered by adding 18 ml. of 0.5 M boric acid and 1.0 ml. of 0.5 M sodium hydroxide per liter. The pH of pericardial fluid even after more than 14 hours of perfusion by solution 2 or 36, so buffered, remained at  $7.4 \pm 0.1$ . No harmful effects of the borate ion were apparent.

#### DISCUSSION

For long continued beating of the lobster heart (20 hours or more) perfused *in situ*, the perfusing solution must correspond closely with the inorganic composition of the serum. Sea water is quite unsatisfactory for perfusion, since it contains 540 per cent more sulfate, 477 per cent more magnesium, 39 per cent less calcium, and 9 per cent less potassium than the serum does. A series of synthetic solutions gradually approaching the inorganic composition of the serum were proved to be increasingly satisfactory. Although hearts will beat for several hours on solutions not containing magnesium and sulfate, they will beat much longer (20 hours or more) if those ions are present. Sodium, potassium, calcium, magnesium, sulfate, and chloride ions are all necessary for prolonged normal beating; the critical number of ions being 100, 3, 5, 2-3, 1-2, and 116 respectively. It is not surprising, therefore, that perfusing solutions used by Hogben (1925) for *Homarus vulgaris*, and by Zoond and Slome (1928) for *Palinurus lalandii*, allowed normal beating for only 3 to 4 hours. For short periods of normal beating the lobster heart will tolerate improperly balanced solutions, but sooner or later the frequency, tone, or amplitude will be altered. Lithium cannot replace sodium; neither can iodide or bromide replace chloride.

Variations in the osmotic pressure of properly balanced solutions up to 15 per cent cause no significant change in the beat, although smaller variations will cause changes if the ionic ratios are not correct.

All of the results on perfusing the lobster heart agree qualitatively with

those on the crayfish heart (Cole, Helfer, and Wiersma, 1939), although the former is quantitatively less sensitive to variations in osmotic pressure, in potassium and in calcium content, but more sensitive to variations in pH than the crayfish heart. These differences may be associated with the differences between sea water and fresh water. The former has a more nearly constant pH, a larger potassium and calcium content than fresh water, but a more variable osmotic pressure, since bay and estuary waters may contain from 275 to 500 millimoles of chloride per liter.

#### SUMMARY

1. An inorganic perfusing solution for the heart of the lobster *Homarus americanus*, to allow prolonged normal beating (20 hours or more) must agree closely with the inorganic composition of the serum, which varies differentially with that of the environmental sea water.

2. All of the chief inorganic ions of the serum are necessary—Na, K, Ca, Mg, Cl, and  $\text{SO}_4$ ; the critical numbers of the ions being 100, 3, 5, 2–3, 116, and 1–2 respectively. Absence of Mg and  $\text{SO}_4$  will be tolerated for several hours.

3. The pH of the solution must agree with that of the serum within 0.2.

4. The osmotic pressure of the solution must agree with that of the serum within 15 per cent.

5. Beating of the heart will continue for several hours on improperly balanced solutions but changes in frequency, tone, or amplitude will occur. Hearts adapted to such solutions will show different responses to physical and chemical stimuli of the solution than those perfused on properly balanced solutions.

6. Arrest in systole is caused by isotonic NaCl, KCl, LiCl, and urea, and arrest in diastole by isotonic  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ , NaBr, NaI,  $\text{MgSO}_4$ , and glucose.

7. Lithium cannot replace sodium; neither can bromide or iodide replace chloride ions.

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# THE STRUCTURE OF THE COLLODION MEMBRANE AND ITS ELECTRICAL BEHAVIOR

## II. THE ACTIVATED COLLODION MEMBRANE

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### I

In preceding communications<sup>1, 2</sup> we were led to the conclusion that the electrochemical activity of collodion membranes, as manifested by concentration potentials, etc., is due principally to acidic impurities. Accordingly, different brands of collodion differ widely as to their activity, the purer brands being less active. The impure (but active) foreign brands of collodion, heretofore generally used by workers in the field of electrochemical membrane investigation, are no longer obtainable. In order to continue our investigation, it became necessary to find methods to produce active collodion membranes at will.

The idea of inducing changes in the electrochemical characteristics of membranes is not entirely new. Many investigators have activated membranes by the adsorption of proteins, e.g. the proteinized membranes of Loeb.<sup>3</sup> Other investigators use other organic compounds, usually dyestuffs. These may be adsorbed like proteins or they may be dissolved in the collodion solution<sup>4</sup> previous to casting the membranes. Such membranes are interesting and useful in their own right, but are not altogether satisfactory substitutes for active collodion membranes. They very often show considerable asymmetry; moreover, the dyestuffs so far employed (according to the literature) are slowly released into the solution in contact with the membrane, whereby the character of the membrane is considerably changed. Meyer and Sievers<sup>5</sup> used an oxidation method to activate a cellophane membrane. Oxidation obviously increases the number of dissociable groups (carboxyl groups) in the membrane. As far as we know, this particular point was not studied further by these inves-

<sup>1</sup> Sollner, K., and Abrams, I., *J. Gen. Physiol.*, 1940, **24**, 1.

<sup>2</sup> Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **24**, 467.

<sup>3</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, **2**, 577, and many subsequent papers in the same *Journal*.

<sup>4</sup> Mond, R., and Hoffman, F., *Arch. ges. Physiol.*, 1928, **220**, 194; Harkewitsch, N. K., *Kolloid-Z.*, 1929, **47**, 101.

<sup>5</sup> Meyer, K. H., and Sievers, J.-F., *Helv. Chim. Acta*, 1936, **19**, 665.

tigators. Recently, Sollner and Abrams<sup>1</sup> have reported in a preliminary paper that the oxidation method can successfully be applied to collodion membranes.

At present we are interested primarily in having available collodion preparations which yield membranes capable of reproducing at least the effects (concentration potentials,<sup>6</sup> anomalous osmosis,<sup>7</sup> etc.) described by previous authors. Moreover, artificial activation of collodion membranes may provide a means of correlating certain empirical data with some more recent theoretical considerations concerning the problem of electrochemical membrane behavior (Meyer and Sievers,<sup>8</sup> Teorell<sup>9</sup>). According to these authors and our own experimental results, the factor determining the electrochemical activity of membranes is—*ceteris paribus*—the number of ionizable groups fixed immovably to the collodion per unit area; *i.e.*, the potential charge density of the collodion-water interface. This charge density is obviously identical with the base exchange capacity of the interfaces. Since this particular phase of the membrane problem has not been adequately developed, we plan to discuss it in a separate paper. The present communication deals with the technique of preparing activated membranes by the oxidation method and with a description of the characteristics of such membranes.

## II

*The oxidation method* can be used in several ways to obtain active collodion membranes. Three possibilities are easily seen: (1) the oxidation of membranes as such, (2) the oxidation of commercial collodion cotton, and (3) the oxidation of cellulose before its nitrification.

The first and second of these methods may be easily applied in the laboratory; they yield satisfactory results quite readily. We have not, therefore, attempted

<sup>6</sup> Michaelis, L., and Fujita, A., *Biochem. Z.*, Berlin, 1925, **158**, 28; 1925, **161**, 47; 1925, **164**, 23; Michaelis, L., and Dokan, S., *Biochem. Z.*, Berlin, 1925, **162**, 258; Michaelis, L., and Hayashi, K., *Biochem. Z.*, Berlin, 1926, **173**, 411; Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol.*, 1926-27, **10**, 575; Michaelis, L., McEllsworth, R., and Weech, A. A., *J. Gen. Physiol.*, 1926-27, **10**, 671; Michaelis, L., Weech, A. A., and Yamatori, A., *J. Gen. Physiol.*, 1926-27, **10**, 685; Michaelis, L., *Bull. Nat. Research Council*, No. 69, 1929, 119; *Kolloid-Z.*, 1933, **62**, 2, and other publications.

<sup>7</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, **1**, 717; 1919-20, **2**, 173, 255, 387, 563, 577, 659, 673; and many other papers in the succeeding volumes of the same *Journal*; Bartell, F. E., and Madison, O. E., *J. Physic. Chem.*, 1920, **24**, 444; Bartell, F. E., and Carpenter, D. C., *J. Physic. Chem.*, 1923, **27**, 101, 252, 346; Bartell, F. E., Membrane potentials and their relation to anomalous osmosis, in Mathews, J. H., Colloid symposium monograph, Department of Chemistry, University of Wisconsin, Madison, 1923, **1**, 120; and many other publications; Preuner, G., and Roder, O., *Z. Elektrochem.*, 1923, **29**, 54; Girard, P., *Compt. rend. Acad. sc.*, 1908, **146**, 927.

<sup>8</sup> Meyer, K. H., and Sievers, J.-F., *Helv. Chim. Acta*, 1936, **19**, 649, 665.

<sup>9</sup> Teorell, R., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 282.

the third possibility—oxidation of cellulose with subsequent esterification to nitrocellulose, as such a procedure would be much less convenient.

Oxidation of cellulose is always accompanied by some degradation, *i.e.* a lowering of the mean molecular weight, mainly by splitting of the chain molecules. The same is undoubtedly true for cellulose derivatives (*e.g.*, collodion). Too thorough oxidation may easily reduce the molecular weight to such an extent that the material involved does not yield membranes of sufficient strength for the intended investigations.

In oxidizing collodion *membranes*, one should be able to find experimental conditions under which the mass of the collodion making up the membrane is relatively little affected, whereas its surfaces, outer as well as inner, are rather highly oxidized. Since the activity of a membrane is obviously determined only by the degree of oxidation at the surfaces, one should be able to produce in this way highly active membranes of relatively great mechanical strength.

If membranes prepared from oxidized *bulk collodion* are to have an appreciable activity, the oxidation process must be carried much further. For this reason, if the oxidized collodion fibres are dissolved, the activated groups originally present mainly at (or near) the oxidized surfaces are distributed uniformly throughout the mass of the collodion. Therefore, if membranes are now cast, the number of activated groups per unit of area in the newly formed surfaces is much smaller than it was in the directly oxidized surfaces of the bulk material prior to dissolution. An appreciable concentration of activated groups in the surfaces of membranes prepared from solutions of bulk oxidized material can, therefore, be obtained only if the latter has undergone a most thorough oxidation.

The method of activating bulk collodion has the obvious advantage that relatively large quantities of material may be prepared in a single operation and that many active membranes can be prepared in the usual manner from the same solution.

For the oxidation of collodion, a number of different oxidizing agents were employed. Among the less effective ones were hydrogen peroxide, bromine water, and potassium permanganate. Sodium hypochlorite, calcium hypochlorite, and particularly sodium hypobromite were found to be effective. These oxidizing agents can, as a matter of course, be applied successfully to other cellulose derivatives and to cellulose, as shown by Meyer and Sievers for the case of cellophane.

In addition to these general oxidation methods, it was found that treatment with alkali was quite effective in producing activated membranes. This observation at first seemed strange; however, after consulting the literature, it was found that nitrocellulose in contact with alkaline solutions is not hydrolyzed in a straightforward manner to cellulose and nitric acid; rather, it undergoes a gradual decomposition of a complicated nature. Nitrite is formed in large



quantities and the nitrocellulose is gradually oxidized. Actually, prolonged action of alkali destroys the material completely with the formation of low molecular weight compounds, particularly acids. A detailed discussion of the mechanism of the reaction of alkalis with nitrocellulose is obviously outside the scope of our present investigation and the interested reader must be referred to the literature.<sup>10</sup>

We have tested the above mentioned effective oxidizing agents over wide ranges of concentration, pH, and reaction times.

The sodium hypochlorite solutions were prepared from commercial solutions by diluting with distilled water. These weakly alkaline solutions were adjusted to the desired pH by addition of hydrochloric acid. Here, as in the forthcoming instances, pH was determined with a glass electrode.

Calcium hypochlorite solutions were prepared by adding 50 gm. of commercial bleaching powder to 1 liter of water. The bleaching powder dissolves only partially, yielding a milky solution. The pH of the originally alkaline solution was adjusted by the addition of hydrochloric acid as before. After the insoluble material settled out, the more or less clear supernatant liquid was used as the oxidizing solution.

The sodium hypobromite solutions were prepared by the addition of molecular bromine to sodium hydroxide solutions. By adding different amounts of bromine, solutions of different pH are readily obtained. Solutions at pH 11 are slightly yellow; at pH 7, orange; at pH 6, deeply red. Saturation is reached at about 5.8. For the best and most consistent results, the solutions should be freshly prepared. In this paper, we characterize our solutions by referring to the concentration of the sodium hydroxide solutions used and the pH rather than by indicating the concentration of hypobromite, the latter being poorly defined and dependent upon hydrogen ion concentration.

The alkaline treatment of our materials was in most cases effected with sodium hydroxide solutions of different concentrations. Potassium hydroxide seems to be about equally effective. Ammonia, on account of its smaller degree of dissociation, is much less effective. The efficiency of other alkalis was also ascertained, but none of them offers any particular advantage over sodium hydroxide.

Our results show that the optimum conditions for the oxidation of bulk collodion are vastly different from those for membranes. But even membranes themselves require varying treatments according to their porosity. In the following paragraphs, we give short descriptions of the best procedures of oxidation which have been found to date. Other collodions may require some adjustment of time or concentration if membranes of the highest activity are desired.

<sup>10</sup> See particularly Schwalbe, C. G., *Die Chemie der Cellulose*, Berlin, Borntraeger, 1910-11 and 1918, pp. 293ff.; also Silberrad, O., and Farmer, R. C., *J. Chem. Soc.*, London, 1906, 89, 1759; and others.

## III

*The oxidation of membranes* is carried out by immersing them for a certain time in the oxidizing solution. This treatment is followed by thorough washing. All operations were carried out at room temperature. To obtain optimum results with any given oxidizing agent, it is necessary to adjust properly the concentration, pH, and the reaction time. Immersion for excessively long periods in a really efficient solution may destroy the membranes. Higher concentrations require somewhat shorter reaction times.

With highly *dried membranes*, bleaching powder solutions were very ineffective, even when allowed to react for several days. Oxidation with sodium hypochlorite solution results in some activation, but in no case were potentials approaching the maximum observed. 0.1 M sodium hydroxide solution yields some increase in activity; stronger solutions have a great tendency to destroy the membranes. However, really satisfactory activation of highly dried membranes was never obtained with sodium hydroxide. Sodium hypobromite solutions in the slightly acid range (*ca.* pH 6) give consistently good results; alkaline solutions beyond a pH of 7 are too destructive to be useful. Though more dilute solutions are quite effective, the best results were obtained by saturating 0.5 M or 1.0 M sodium hydroxide solution with bromine. (The use of more concentrated solutions has no advantage.) These solutions, if freshly prepared, give consistently satisfactory results, the proper immersion time being about 3 hours. We recommend this last method for the activation of dried collodion membranes as the one most consistently satisfactory.

*Porous membranes* having a fair degree of activity may be obtained with solutions of sodium hypochlorite and calcium hypochlorite over a wide range of hydrogen ion concentration (pH 6–11.5). The activation is stronger when alkaline solutions are employed, but above a pH of about 10.5, there is much danger of the membranes being destroyed. Sodium hydroxide in concentrations above 0.02 M is suitable in activating porous membranes; the reaction time has to be chosen carefully to fit the concentration. For example, immersion for 45 minutes in 0.02 M sodium hydroxide yields membranes of about the same degree of activity as treatment for 5 to 10 minutes with 0.5 M solution. The activity obtained is only slightly better than that which results from oxidation with hypochlorites.

With porous (as well as with the dried) membranes, entirely satisfactory results were obtained consistently with sodium hypobromite solutions. The optimum pH range, however, was somewhat different, being between 7.0 and 9. Acid solutions are somewhat less effective; more alkaline solutions are too destructive. After some testing, we recommend the use of 0.5 M to 1.0 M sodium hydroxide solution with the addition of sufficient bromine to adjust the pH to a value between 7 and 9. The optimum oxidation time in such solutions was 3 to 4 hours for the membranes we usually used (the thickness of our porous

membranes being on the average 0.04–0.05 mm.). Thicker membranes may stand much longer oxidation without damage. Therefore, the likelihood of obtaining more highly activated membranes is greater with somewhat thicker films.

The changes in geometrical structure (porosity) which the membranes may undergo during oxidation will be discussed below after the presentation of the pertinent experimental data.

The physical properties of oxidized membranes are essentially the same as those which have not been treated. Moderately oxidized membranes are nearly as strong mechanically as those which are unoxidized. Increasing activation weakens them more and more; this is accompanied, particularly with porous membranes, by the appearance of an increasing degree of "plasticity," a property which is practically absent in unoxidized membranes.

#### IV

Various methods of testing the *electrochemical activity of membranes* were used according to their porosity. In the case of highly dried membranes, the only practical method is the determination of the concentration potential (Michaelis) before and after oxidation. For highly porous membranes, the most sensitive method is to determine the extent of anomalous osmosis under standard conditions. In addition, we measured electroosmosis as well as concentration potentials before and after oxidation. We also determined the influence of oxidation on the rate of filtration and on the pore volume.

The effect of oxidation on three representative *dried membranes* is summarized in Table I.

The membranes were cast from 5 per cent Mallinckrodt "Parlodion" solution (75 per cent absolute ether, 25 per cent absolute alcohol) and dried for 24 hours. The concentration potentials for 0.1 M KCl/0.01 M KCl were then determined. After this, the membranes were oxidized for about 3 hours with sodium hypobromite solution, prepared by saturating molar sodium hydroxide with bromine. They were thoroughly washed with water to remove all traces of electrolyte and the concentration potentials again measured.

The values obtained after oxidation closely approach the thermodynamically possible maximum of about 56 mv. (at 25°C.), indicating an unusually high degree of ionic selectivity. The ohmic resistance of these membranes is very markedly decreased after oxidation.

In testing *porous membranes*, it was necessary to compare only membranes of the same general porosity characteristics. With some experience, it is not difficult to produce such membranes. One must use some good grade of collodion dissolved in a standard solvent mixture; each time a membrane is cast, the same procedure has to be followed.

Commercial solutions of Baker or Merck "Collodion U.S.P." (5 per cent collodion in a mixture of 25 per cent alcohol and 75 per cent ether) were used for most of the following experiments; occasionally, we employed collodion cotton (Baker "Pyroxilin") or Mallinckrodt "Parlodion" (5 per cent dry material in 25 per cent absolute alcohol and 75 per cent absolute ether). The differences between these preparations are negligible for our present purposes.

The technique of preparing the membranes, as described previously, is briefly as follows: Collodion bags are cast in  $30 \times 110$  mm. test tubes and allowed to dry several minutes. Next the bags are filled with water; they loosen from the glass spontaneously and are tied to glass rings with thread while still filled with water. Following this, they are kept in covered glass containers under water to which thymol has been added as a preservative. They are never allowed to dry. The membranes so prepared are fitted to rubber stoppers provided with a long capillary tube (inner diameter about 1.5 mm.). Those membranes were selected which, when filled with

TABLE I

*The Influence of Oxidation on the Concentration Potential across Dried Collodion Membranes*  
—  $KCl\ 0.1\ M/KCl\ 0.01\ M$  +

*The Sign is That Observed in the External Circuit*

Membrane No.	Concentration potential	
	Before oxidation	After oxidation
	mv.	mv.
1	16.8	53.5
2	25.3	53.7
3	11.0	53.3

0.25 molar sucrose solution and placed in water for 20 minutes, yield an osmotic rise ("sucrose value") of about 120 mm. of liquid in the capillary manometer. The adjustment of the zero reading is facilitated by a small glass syphon provided with a stopcock, allowing the rapid adjustment of the meniscus in the manometer to the proper level, corresponding to the capillary rise over the outside solution.

These membranes are very suitable for experiments concerning anomalous osmosis. Their water content varied only in rather narrow limits between 65 and 70 per cent by weight. They were rather thin, their average thickness being 0.04 to 0.05 mm., with appreciably thinner and thicker parts found on each individual membrane.

To test a membrane for anomalous osmosis, the collodion bag is filled with salt solution, our standard being  $\frac{M}{512}$  potassium sulfate. The bag was then fitted with stopper and manometer tube and immersed in a beaker filled with pure water. The rise of the meniscus in the manometer tube after 20 minutes is taken as the measure of the extent of anomalous osmosis. This rate of rise is very characteristic for each membrane.

Table II shows the effect of alkaline treatment on porous membranes after different reaction times; the values in Table III were obtained with membranes oxidized in molar sodium hypobromite solution (pH 7.1).

TABLE II

*The Activation of Porous Collodion Membranes with 0.5 M Sodium Hydroxide Solution*

Membrane	Before oxidation		Duration of treatment with sodium hydroxide	After oxidation		
	Osmotic rise with sucrose 0.25 M	Anomalous osmosis with $\frac{M}{512}$ $K_2SO_4$		Osmotic rise with sucrose 0.25 M	Anomalous osmosis with $\frac{M}{512}$ $K_2SO_4$	Mechanical strength of membrane
	mm.	mm.	min.	mm.	mm.	
a	108	34	1	108	100	Very strong
b	145	53	2	143	178	" "
c	132	42	3	136	155	" "
d	110	40	5	105	223	Strong
e	122	54	10	128	290	Weak
f	116	38	15	118	284	Very weak
g	98	32	20	120	295	" "
h	110	44	60	—	—	Broken

TABLE III

*The Activation of Porous Collodion Membranes\* with 1 M† Sodium Hypobromite Solution (pH 7.1)*

Membrane	Before oxidation		Duration of oxidation	After oxidation	
	Osmotic rise with sucrose 0.25 M	Anomalous osmosis with $\frac{M}{512}$ $K_2SO_4$		Osmotic rise with sucrose 0.25 M	Anomalous osmosis with $\frac{M}{512}$ $K_2SO_4$
	mm.	mm.	hrs.	mm.	mm.
a	111	43	4	132	312
b	128	46	4	153	346
c	146	58	4	148	356
d	147	55	18	164	352

\* The membranes used in this experiment were somewhat thicker than those we used ordinarily (see text).

† Prepared from 1 M sodium hydroxide solution, therefore actually about 0.5 M with respect to hypobromite ion.

The effect of alkaline treatment on the sucrose values is conspicuously small, though the activation as measured with anomalous osmosis is rather good. It seems that a limiting degree of activation is reached after a few minutes. The membranes which were immersed for less than 10 minutes showed great mechanical strength. The last three became progressively weaker. We have here an example of thorough activation of collodion membranes with a minimum change in spatial structure.

Such values as those given in Table III can be obtained quite regularly on oxidation with sodium hypobromite. This table also exemplifies the changes in behavior towards nonelectrolyte solutions which are generally observed. A graphic presentation of the effect of oxidation on anomalous osmosis has recently been published.<sup>1,11</sup> Additional data are also contained in Table VII.

At this point, a brief discussion of the changes in the geometrical structure (porosity) which the membranes undergo on oxidation may be in order. Such changes may well be indicated by their behavior towards nonelectrolyte solutions. The observation of only very slight variations in the osmotic behavior of nonelectrolytes (see tables below and some previously published curves<sup>1</sup>) has led us to conclude that the geometrical structure of our membranes is but little affected by oxidation. The experimental data indicate that these changes increase with longer oxidation times. Limited (minutes only) treatment with sodium hydroxide solutions and the oxidation with acid hypobromite, particularly the latter, seem to combine good activation with minimum changes in porosity. Filtration experiments also indicate that the changes in the geometrical structure of the membranes on oxidation are small. For example, filtration rates through three of our typical membranes previous to oxidation were 2.03, 2.38, and 2.05 ml. per hour under a hydrostatic head of 15 cm. water, the filtering area being roughly 100 cm<sup>2</sup>. After oxidation, these values were 1.74, 2.14, and 1.98 ml. per hour. To minimize disturbances which could be caused by electrical forces, molar potassium chloride solution was used in these filtration experiments.

In order to further clarify the question of structural changes caused by oxidation, we performed some experiments with flat membranes having approximately the same porosity as our bag-shaped porous membranes. These membranes were measured as to length, width, and thickness, and their weight in the wet state (blotted with filter paper) was determined before and after oxidation. A loss of volume was detectable only by the weight measurements and this only in certain cases. Immersion of the membranes in 0.5 M sodium hydroxide solution for 10 minutes caused a loss in weight of about 3 per cent; 3 hours in molar sodium hypobromite at pH 9.0 and 7.1 resulted in a loss of about 2 per cent and 0.7 per cent respectively, the latter value being within the limits of

<sup>11</sup> The anomalous osmosis values in these curves are appreciably higher than those in Table III for the following reason. The points of the curves were obtained by performing the experiments one after the other with decreasing electrolyte concentrations without any intermediate rinsing of the membranes with water. Therefore, at the beginning of each experiment, the pores of the membranes contained the electrolyte in even higher concentration than in the bulk of the solution. No time was thus required for its penetration into the membrane. For this reason, the anomalous osmotic liquid movement sets in immediately with full force without any initial latent period.

experimental error. The percentage of dry material of the membranes oxidized with hypobromite did not change significantly. Prolonged treatment, particularly with hydroxide, results in a considerable loss of material and change in porosity.

TABLE IV

*The Influence of Oxidation on the Concentration Potential across Porous Collodion Membranes*

Membrane	Before oxidation			After oxidation		
	Osmotic rise with sucrose 0.25 M	Concentration potentials*		Osmotic rise with sucrose 0.25 M	Concentration potentials*	
		KCl 0.1/0.01 M	KCl 0.2/0.1 M		KCl 0.1/0.01 M	KCl 0.2/0.1 M
	mm.	mv.	mv.	mm.	mv.	mv.
a	122	2.8	0.1	140	26.2	4.9
b	125	2.4	0.3	160	18.8	2.0

\* The dilute solution was positive in the external circuit.

TABLE V

*The Influence of Oxidation on the Electroosmosis through Porous Membranes (Current 10 m Amps.)*

Membrane	Concentration of KCl solution	Electroosmotic flow in 20 minutes	
		Before oxidation*	After oxidation*
	moles/liter	c.mm.	c.mm.
a	0.001	2420	3750
	0.005	730	2300
	0.01	360	1210
	0.05	0	490
	0.1	0	360
b	0.001	1690	2780
	0.005	1090	3500
	0.01	600	1930
	0.05	0	120
	0.1	0	—

\* Osmotic rises with sucrose solution as listed in Table IV.

The evidence presented indicates that careful oxidation does not cause major changes in the geometrical structure of collodion membranes, though minor alterations probably cannot be avoided entirely. These changes, however, are certainly much smaller than the differences found between different membrane specimens cast by hand under as constant conditions as possible.

The influence of oxidation on the dynamic membrane (concentration) potential of porous membranes is indicated by the data in Table IV. Various

concentrations of potassium chloride were used. Results obtained with sulfate and citrate solutions are similar. The membranes used for these experiments were cast from a 5 per cent solution of Mallinckrodt "Parlodion"; they were of the same porosity as those used for experiments on anomalous osmosis.

Table V summarizes the results of some electroosmosis experiments carried out with the same membranes as those in Table IV.

For the electroosmosis experiments, membranes secured to glass rings were fitted with a rubber stopper holding a 6 mm. wide glass tube. One electrode consisted of a platinum wire which protruded down through this tube. The other electrode was in the form of a platinum wire cage surrounding the membrane. The membrane was filled with an electrolyte solution and placed in a bath of the same solution. The current in all experiments was kept constant at 10 milliamperes by manual adjustment, the effective membrane area being about 100 cm<sup>2</sup>. or slightly less. Before the actual measurements were made, the current was passed through the membranes for 10 minutes. After this, the zero position of the water meniscus in the glass tube was marked. A current of 10 milliamperes was now passed through the system for 20 minutes and the change in the position of the meniscus noted. From this change and the diameter of the tube, the electroosmotic transport is easily calculated. The quantities transported electroosmotically are directly proportional to the duration of the experiment and to the current applied, back filtration being negligible. Prolonged passage of the current through the membranes, particularly activated membranes, weakens them appreciably. They may be easily destroyed when strong currents are applied over a long period of time. The alkali developed at the membrane when the current passes<sup>12</sup> is probably responsible for its destructive action.<sup>13</sup>

The marked influence of oxidation is apparent. As one should expect from theoretical considerations, the relative effect of activation is more pronounced with the more concentrated electrolyte solutions.

## V

*The oxidation of collodion in bulk* is performed by immersing dry<sup>14</sup> fibrous collodion cotton in proper oxidizing solutions. After a suitable oxidation time,

<sup>12</sup> Bethe, A., and Toropoff, T., *Z. phys. Chem.*, 1914, **88**, 686; 1915, **89**, 597.

<sup>13</sup> This destruction (previously described for highly dried membranes by Michaelis, (*Bull. Nat. Research Council*, No. 69, 1929, 135), is accompanied by the dissolution of some of the destroyed material in the solution. Therefore, the use of collodion membranes for electrodialysis seems much less advisable than is generally assumed, particularly when very high degrees of purity are required. We are inclined to believe that the generation of soluble impurities may have seriously impaired some of the published work on highly purified inorganic colloids.

<sup>14</sup> Though collodion is generally not explosive, great quantities of dry material should never be kept in the laboratory. The dry material should always be handled with due care.



the material is washed thoroughly with dilute hydrochloric acid and liberal quantities of distilled water. After this, it is dried <sup>14</sup> in the open at moderately increased temperature. It is finally dissolved in ether-alcohol; 5 per cent solutions were used.

The material which may be most conveniently oxidized is collodion cotton<sup>15</sup> (native nitrated cellulose fibres). It can be readily obtained from supply houses. It may also be reprecipitated collodion, obtained in the fibrous form by slowly dripping commercial collodion solutions into a great excess of violently stirred distilled water. For the subsequent oxidation, it makes little difference whether native or reprecipitated material is used.

Here, as with the membranes, we tried several oxidizing agents for varying reaction times at different pH. The oxidizing solutions used were the same as those described above. As before, the more alkaline solutions of hypochlorite and hypobromite are markedly more effective than those of lower pH. For reasons outlined above, the oxidation has to be much more thorough in the case of bulk collodion than with membranes.

Immersion of collodion cotton in sodium hypochlorite or hypobromite solutions at pH 6 to 10 for 2 days yielded only moderately active products; treatment for 16 hours at pH 11 to pH 11.5 was more effective. But the loss of material increases very markedly in the more alkaline solutions. Both sodium hypochlorite and hypobromite are less effective activating agents than bleaching powder (calcium hypochlorite) solutions. Treatment for 2 days with such solutions at pH 8 to pH 11.5 gives comparatively good activation. The yield is good, particularly if the reaction is carried out at a pH below 10.

The simplest method of oxidation is the treatment with sodium hydroxide. 0.02 M solution for 5 days is nearly as effective as 0.1 M for 2 days, 1 M for 30 minutes, or 2 M for 15 minutes. The loss of material under these conditions is moderate (less than 10 per cent). The activation of bulk collodion with hydroxide is at least as effective as any other method previously described. Membranes cast from such material are quite strong, though oxidation is always accompanied by some lowering in mean molecular weight, as evidenced by the somewhat lower viscosity of the solutions.

By carefully adjusting the conditions, one can obtain reasonably well activated collodion practically without any loss in mechanical strength (and of material). Too thorough oxidation, apart from the loss of material, results in a product of poor mechanical properties. Its solutions have a very low viscosity and membranes cast from them are somewhat plastic and weak, often too weak to be useful for experimental purposes. For this reason, the activation of collodion cannot be carried successfully beyond a certain point. The introduction of a maximum number of ionizable groups into the nitrocellulose

<sup>15</sup> Collodion cotton (e.g., Baker "Pyroxilin") comes moistened with alcohol. The alcohol must be allowed to evaporate before the above treatment is begun.

molecule is limited by the ability of the partially decomposed product to form mechanically adequate membranes.

With the oxidized collodion it is advisable, though not absolutely necessary, to use as solvent ether-alcohol mixtures of somewhat higher alcohol concentration than that used in commercial U.S.P. solutions (see above). We frequently used 35–40 per cent alcohol and 65–60 per cent ether. For the preparation and properties of dried membranes, the solvent composition, as it seems, is without significance; the preparation of porous membranes of any particular porosity may be greatly facilitated by use of the proper solvent mixture. However, for the time being, we would like merely to mention the solvent influence without discussing it further.

The activation of collodion in the dissolved state was also attempted. This reaction proceeds very rapidly and is difficult to control. Therefore, this method yielded rather indifferent results; very pronounced degradation was accompanied by only moderate activation.

For the activation of bulk collodion for general experimental purposes, we recommend a short (about 20 minutes) treatment with 1.0 *M* sodium hydroxide solutions. An alternative method involves the use of bleaching powder (50 gm./liter) solution in the slightly alkaline range (pH 8–10) using long (2 days) oxidation periods. It is advisable to use a sufficiently large excess of oxidizing solution (e.g., 1 liter molar sodium hydroxide solution for 50 gm. collodion). In this way, any desired quantity of activated collodion can easily be prepared and large numbers of membranes can thus be cast from the same material.<sup>16</sup>

## VI

*Dried membranes* prepared from properly activated collodion regularly give concentration potentials (0.1 *M* KCl/0.01 *M* KCl) of 52 to 54 mv. These values are appreciably higher than those formerly obtained with even the most active commercial—now unobtainable—preparations. The concentration potentials of such membranes (using 0.01 *M* KCl/0.001 *M* KCl) vary between 56 and 58 mv. at room temperature. The ohmic resistance of such membranes is much lower than that of similar membranes prepared from untreated material. This effect seems to be particularly pronounced with membranes prepared from

<sup>16</sup> Concerning additional methods of activating collodion membranes, we should like to mention that some casual attempts to produce sulfuric acid esters by treating collodion with sulfuric acid gave negative results. In some other experiments, we mixed some nitropectic acid with inactive collodion and obtained very active membranes. According to the literature (Henglein, F. A., and Schneider, G., *Ber. chem. Ges.*, 1936, 69, 309), nitropectin (and nitropectic acid), when properly prepared, yields films similar to those of collodion. Such films of pure nitropectic acid should, as our experiments indicate, be electrochemically active.

collodion thoroughly activated with sodium hydroxide. We hope to be able to investigate this and related points later.

In order to characterize the degree of activity of *porous membranes* prepared from bulk oxidized collodion, some data concerning anomalous osmosis are summarized in Table VI.

Variations between different batches are more sensitively detected by anomalous osmosis measurements with porous membranes than by determinations of concentration potentials on highly dried membranes.

Activity as good as that of samples *a* and *b* in Table VI are rather rare. Collodion having an activity similar to that of samples *c* and *d* can be obtained easily and regularly. It must be emphasized that the activity of our most

TABLE VI

*The Influence of Oxidation on the Activity of Bulk Collodion: Anomalous Osmosis*

Membrane material	Osmotic rise with sucrose 0.25 M	Anomalous osmotic rise with $K_2SO_4 \frac{M}{512}$
	mm.	mm.
Commercial (unoxidized) collodion. ....	110-140	35-55
Oxidized collodion sample <i>a</i> . ....	140	260
Oxidized collodion sample <i>b</i> . . . . .	132	250
Oxidized collodion sample <i>c</i> . . . . .	130	211
Oxidized collodion sample <i>d</i> . . . . .	136	180

active *porous* membranes prepared from bulk collodion does not approach the degree of activation which may be regularly obtained by the oxidation of membranes as such.

## VII

*Membranes prepared from crude collodion* should (according to a preceding paper<sup>2</sup>) be active, since crude preparations are bound to contain acidic impurities. These acidic impurities are formed in the process of manufacture and are removed by proper purification of the commercial preparations. According to the technical literature, crude nitrocellulose is not sufficiently stable for safe handling, at least in the dry state. Consequently, manufacturers are generally reluctant to release any material in the crude state. Finally, however, we procured a sample of crude collodion from the Monsanto Chemical Company.<sup>17</sup> When dissolved (in a mixture of 65 per cent ether and 35 per cent alcohol), its solutions were turbid and yielded a sediment on centrifuging. (Only centrifuged solutions were used.) The activity of membranes prepared from these

\* <sup>17</sup> For this sample we are greatly indebted to Mr. H. K. Nason, Assistant Director of Research, Monsanto Chemical Company, Springfield, Massachusetts.

solutions, when tested by means of anomalous osmosis, was somewhat higher than the most active Schering-Kahlbaum preparation; on the other hand, they were definitely less active than most membranes prepared from collodion oxidized as discussed in the preceding paragraphs. Highly dried membranes gave values for the concentration potential ( $\text{KCl } 0.1 \text{ M}/\text{KCl } 0.01 \text{ M}$ ) of 50 mv. and better. These values are similar, or possibly somewhat higher, than those reported by Michaelis and coworkers, who used membranes prepared from Schering-Kahlbaum "Celloidin."

Since crude collodion is not regularly available through supply houses or other regular trade channels, it seems superfluous to describe this product or its behavior in detail.

### VIII

The results obtained with the oxidative activation of membranes lead one to infer that the differences observed in the *membranes prepared from various brands of collodion* (reported previously<sup>1,2</sup>) would disappear on oxidation. The verification of such an inference would be of importance in providing an insight into the structure of collodion membranes.

The best way to approach the above problem experimentally is to test the behavior of such membranes with anomalous osmosis. Table VII summarizes the behavior of membranes prepared from several brands of collodion before and after oxidation. The experimental technique was the same as that previously sketched.

In order to have reasonable uniformity in the membranes prepared from the different collodions,<sup>18</sup> we selected those which gave as nearly as possible the same rate of pressure rise when tested with 0.25 M sucrose solution (Table VII a column 2). The pressure rise obtained during 20 minutes was taken as indicative of the porosity of the membrane. The rises of the liquid in the manometer tube after 20 minutes with different electrolytes are given beside the sucrose values (Table VII a columns 3, 4, and 5). The values given here were obtained in "repeat" experiments, carried out without washing the membranes after a preceding similar experiment with the same electrolyte concentration. These repeat values are always somewhat higher, as the electrolyte has already penetrated the membrane and the proper concentration gradient is established from the very beginning of the experiment. The electrolyte con-

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<sup>18</sup> To obtain such structural uniformity with the different brands of collodion, it was necessary to evaporate the original Schering-Kahlbaum collodion solutions to dryness and to redissolve the dry material in ether-alcohol prior to casting the membranes. For the experiments described in Table VII, we adhered to the standard solvent mixture (75 per cent ether and 25 per cent alcohol) and made the solutions uniformly 5 per cent. In parenthesis, we may add that in later experiments "Parlodion" membranes having a satisfactory porosity were obtained only by increasing the alcohol concentration to 40 per cent.

centrations used are approximately optimum concentrations for membranes of moderately good activity. After being thus tested, the membranes were oxidized for 3 hours in a solution prepared by saturating molar sodium hydroxide with bromine, the resulting solution having a pH of about 5.8. At this pH, the activation is good, but not necessarily optimal. After properly washing the membranes, the osmotic experiments with sucrose and electrolyte solutions were repeated.

The results are shown in Table VII b. It may be noted that on account of the poor quality of Schering-Kahlbaum DAB 6, we were unable to obtain a membrane of this material which withstood oxidation without damage.

TABLE VII

*The Influence of Oxidation on the Activity of Porous Collodion Membranes, Prepared from Various Brands of Collodion: Anomalous Osmosis*

1	2	3	4	5	6	7	8	9
Brand of collodion	a				b			
	Before oxidation				After oxidation			
	Osmotic rise with sucrose 0.25 M	Anomalous osmosis			Osmotic rise with sucrose 0.25 M	Anomalous osmosis		
		$\frac{M}{256} \text{ KCl}^*$	$\frac{M}{512} \text{ K}_2\text{SO}_4^*$	$\frac{M}{128} \text{ K}_2\text{ citrate}^*$		$\frac{M}{256} \text{ KCl}^*$	$\frac{M}{512} \text{ K}_2\text{SO}_4^*$	$\frac{M}{128} \text{ K}_2\text{ citrate}^*$
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
Mallinckrodt "Parlodion".	124	7	43	75	142	112	332	665
Merck collodion U.S.P. ....	128	14	54	112	140	124	290	650
Baker collodion U.S.P. ....	128	6	48	130	120	116	338	670
Schering-Kahlbaum Collodion "pro analysi" ....	125	8	32	124	140	104	320	715
Schering-Kahlbaum Collodion "zur Herstellung von Membranen" ....	115	16	83	190	145	75	173	620
Schering-Kahlbaum "Celloidin" "for general use".	126	70	228	410	158	117	340	635
Schering-Kahlbaum Collodion DAB 6. ....	124	26	195	390	Decomposes on oxidation			

\* This concentration was chosen because it gives maximum effects with membranes of medium activity.<sup>1</sup>

A comparison of the pressure rises with sucrose solution (obtained with the same membranes) before and after oxidation shows that they are but little changed (Table VII, columns 2 and 6). This is particularly true for the better (less active) grades of collodion. The behavior of our membranes with electrolyte solutions, however, is most markedly changed after oxidation. They are all uniformly active, possessing far greater activity than even the most active commercial preparations. The differences between the various commercial preparations completely disappear on oxidation, a fact which indicates that membranes prepared from different collodions (if they behave fairly uniformly with nonelectrolytes) have a fairly similar geometrical structure. Thus the difference between membranes prepared from different brands of

collodion is to be found not in differences of their geometrical structure, but rather in their different electrochemical characteristics.

## IX

### DISCUSSION

Most of the points raised by the data which have been presented have been dealt with adequately in the foregoing sections. Certain other facts warrant further comment and correlation.

It should be said that the proposed methods of activation of collodion by oxidation are obviously not beyond possible improvement. They are simply methods found to be satisfactory with many different collodion preparations under varying conditions.

Concerning the origin of the oxidized groups in the active commercial preparations (as well as in the crude collodion), it may be appropriate to mention that the strength of the hypochlorite (and hypobromite) solutions we used was about the same as that used in technical bleaching solutions; the latter usually contains 2 to 5 gm., occasionally up to 25 gm. of "available chlorine" per liter—according to the material to be bleached. Cellulose swells in electrolyte solutions, and the native cellulose fibres are thus quite accessible to the action of bleaching agents. There is little wonder that considerable oxidation occurs during the manufacturing process, particularly if impure raw materials requiring thorough bleaching are used. The latter treatment in combination with the obviously rather superficial purification of the active collodions (as attested by their high ash and sulfate content<sup>2</sup>), can readily account for the activity of certain commercial collodion preparations of poor quality which are no longer available.

The problem as to why one or the other of our oxidizing agents is more or less effective under different conditions probably cannot be solved at present; too many factors are involved. Among the strictly chemical factors are the change of oxidizing power in general with changing hydrogen ion concentration, the different possibilities of attacking the nitrocellulose in an oxidative and hydrolyzing manner, and the correlation of these two processes.

There are other problems in which chemical and structural factors are closely interwoven. For example, a given oxidizing agent may or may not penetrate into the smaller pores of the collodion, depending on its chemical characteristics, particularly its molecular (ionic) size. If the latter is large, it may be unable to reach the narrow pores of a dried membrane, unless it first causes considerable structural changes of a destructive nature.

This leads us to a very important point, namely the possible structural changes which occur on oxidation. We must expect such changes to be much more critical with dried than with porous membranes. The relative structural changes brought about by a certain oxidizing agent may be insignificant in

the wide pores of a porous membrane, whereas the same absolute change in the extremely minute pores of a dried membrane may easily result in proportionately enormous structural changes. Therefore, an oxidizing agent which gives optimum activation with porous membranes may be very unsuitable for dried membranes. For the latter, only such oxidizing agents which cause minimal structural changes (*e.g.*, acid hypobromite solution) are suitable. Such activating agents, although they may not bring about maximum charge density, leave the pores sufficiently narrow to give rise to maximum concentration potentials.

Our own results lead to the conclusion that differences between active collodion membranes and membranes artificially impregnated with proteins, dyestuffs, etc., are not nearly so fundamental as formerly assumed. In all these cases, certain ionizable substances, foreign to the pure ideal nitrocellulose cause the activity.

The idea of increasing the base exchange capacity of cellulosic materials is certainly not new. Years ago, Schwalbe and Becker<sup>19</sup> oxidized cellulose with the purpose of introducing acid groups and studied the base exchange properties of the oxidized products.

A brief explanation of the terms used to describe the activity of our membranes, particularly the porous ones, may be appropriate. Measurements of concentration potentials are basically the simplest way of characterizing dried membranes. In our opinion, simple potential values which are obtained experimentally are hardly less characteristic than any derived constants which may be calculated according to some newer theoretical concepts.<sup>8, 9</sup> In the case of porous membranes, the measurement of concentration potential (or anything derived from it) gives only an incomplete characterization. For a more detailed description of a membrane, potential measurements must be supplemented by some sort of electrokinetic experiments (*e.g.*, electroosmosis measurements). A much more sensitive indicator of the electrochemical "activity" of actual membranes (having pores of different diameters<sup>20</sup>) is, as pointed out before, the extent of anomalous osmosis under standard conditions—as used in our experiments.

Most preceding studies (as well as our own) have neglected a matter of primary importance, namely, the conductivity of collodion membranes. Michaelis and coworkers<sup>21</sup> have investigated this problem to a limited extent. Conductivity measurements, performed with membranes of different activity,

<sup>19</sup> Schwalbe, C. G., and Becker, E., *Ber. chem. Ges.*, 1921, **54**, 545.

<sup>20</sup> Sollner, K., *Z. Elektrochem.*, 1930, **36**, 36, 234; Sollner, K., and Grollman, A., *Z. Elektrochem.*, 1932, **38**, 274; *Tr. Electrochem Soc.*, 1932, **61**, 477, 487; Sollner, K., *Kolloid-Z.* 1933, **62**, 31.

<sup>21</sup> Green, A. A., Weech, A. A. and Michaelis, L., *J. Gen. Physiol.*, 1929, **12**, 473; also, Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol.*, 1927, **10**, 575.

would probably be rather helpful in elucidating their structure. From the experimental point of view, the problem of conductivity is particularly urgent with highly dried membranes. In the past, investigators have been concerned nearly exclusively with the electromotive properties (concentration potentials) of such membranes. By and large, as long as the resistance was not so high as to make measurements too difficult or inaccurate, a membrane was considered to approach perfection if the concentration potential approached the thermodynamically possible maximum. Ordinarily, dried membranes have such high electrical resistance (and low permeability for nonelectrolytes) as to make them more or less useless for many permeability studies. If highly selective membranes should be used for large scale permeability studies or for model experiments (*e.g.*, straight permeability or Donnan equilibria with uni-valent strong electrolytes), they must combine both high ionic selectivity and low resistance. The oxidation method might conceivably offer a good means of preparing such membranes; however, this possibility requires further experimental investigation.

Finally, it seems worth while once more to stress the fact that the oxidation of membranes influences their behavior towards nonelectrolytes only slightly and that their geometrical structure is only little affected by such treatment. Membranes prepared from different brands of collodion completely lose their characteristic differences in behavior towards electrolyte solutions (Table VII) when oxidized (and remain, as implicitly said above, relatively unchanged in their behavior towards nonelectrolytes).

This indicates that the differences between membranes prepared from different brands of commercial collodion in their behavior towards electrolyte solutions have to be sought for primarily in their electrochemical structure, *i.e.* in the number (and nature) of dissociable groups in their surfaces. Particle size and particle size distribution are obviously, at least in the range of the investigated preparations, only a secondary factor.

With this statement, we do not infer that the differences of molecular weight between the different brands of collodion are not important for the spatial structure of the membranes. What we can say on the basis of our present experimental results is that the different commercial collodion preparations allow one to prepare membranes having obviously similar geometrical structures. There are great differences in the ease with which membranes of a given porosity (sucrose value) can be obtained from different collodion preparations, particularly if the same solvent mixture is used.

A deeper insight into the physical and chemical structure of the collodion membrane will be possible only after further experimental data are available. The most important problem seems to be the determination of the absolute charge density at collodion-water interfaces and the possible correlation of such data to the Meyer-Sievers-Teorell theory of electrochemical membrane



behavior. The water uptake and the swelling of collodion membranes in different electrolyte solutions also await investigation. We hope to deal with these problems in subsequent publications.

#### SUMMARY

1. The electrochemical behavior (concentration potential, anomalous osmosis, etc.) of collodion membranes is due to its acidic impurities. These impurities determine the possible charge density of the collodion—aqueous solution interfaces. This (possible) charge density is believed to be identical with the base exchange capacity of the interfaces under consideration.

2. The collodion preparations commercially available at present are too pure to yield membranes of sufficient activity for electrochemical membrane investigations. Crude collodion, a product which is only partially purified, shows considerable electrochemical activity because of its content of acidic impurities.

3. The inactive commercial collodion preparations can readily be activated by oxidation by virtue of the fact that oxidation increases the number of dissociable groups (carboxyl groups) on the collodion.

The oxidation method of activating collodion may be applied to membranes as such as well as to collodion in bulk.

4. The recommended oxidizing agents are sodium and calcium hypochlorite and sodium hypobromite. A further group of effective and recommended activating agents are solutions of strong alkalies. Alkalies cause a complicated decomposition of nitrocellulose with the formation of nitrites (and probably other nitrous compounds). These nitrites act upon the collodion as oxidizing agents, thus causing activation.

5. Detailed descriptions of tested oxidation procedures for highly dried membranes, porous membranes, and bulk collodion are given in the text, the optimum conditions being different in the three cases.

6. Collodion membranes oxidized as such show a much higher electrochemical activity than any previously described. Highly dried membranes after oxidation give concentration potentials which approach the thermodynamically possible maximum more closely than any given in the literature. Porous membranes after oxidation show greatly increased concentration potentials and yield much greater electroosmosis when a current is passed through. These effects are reflected in the enormous magnification of the extent of anomalous osmosis.

7. The behavior of the porous membranes toward nonelectrolytes changes but little on oxidation. The volume of such membranes, as well as their per cent water content (pore space), remains constant within the limits of experimental error. From this observation and studies on the rate of filtration, it is concluded that the geometrical structure of membranes is but little changed on oxidation.

8. Collodion oxidized in bulk likewise yields very active membranes. Dried membranes prepared from activated bulk collodion consistently yield concentration potentials which approach the thermodynamically possible maximum very closely and are appreciably higher than any previously reported. Porous membranes prepared from bulk oxidized collodion show a degree of electrochemical activity surpassing anything described for the most active commercial collodion preparations. However, these membranes are less active than those oxidized as such.

9. Membranes prepared from different collodion preparations which behave fairly uniformly towards nonelectrolytes but very differently towards electrolyte solutions become similar in their behavior towards electrolytes after oxidation.

10. The geometrical structures of membranes prepared from different collodion preparations are essentially identical. The differences in their behavior towards electrolytes are due entirely to the electrochemical factor; *i.e.*, the charge density at the water/collodion interface.

11. Certain general aspects of the foregoing experimental results are discussed briefly.



# RECTIFICATION AND INDUCTANCE IN THE SQUID GIANT AXON

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The function of nerve has been extensively investigated for many years and careful measurements have been made of the initiation and propagation of the impulse under a wide variety of conditions. It was only natural that there should be numerous attempts to correlate and explain these experimental data. The general phenomenological theories of nerve activity have avoided the physical characteristics of nerve structures and the specific theories have had to consider these properties as undetermined parameters. It has long been assumed, for example, that the axon membrane could be represented electrically, by capacity and resistance elements, yet these characteristics have only been measured directly in the past few years. Excitation phenomena have also led to a few suggestions that the membrane might act as an electrical rectifier and to at least one mention of the possibility of an inductive element in a nerve.

The preceding papers have presented experimental evidence, independent of excitation, for the rectification and inductance elements in the membrane of the squid giant axon (Cole and Baker, 1941 *a* and *b*; Cole and Curtis, 1941). The various aspects of the evidence should now be correlated and the implications as to both the physical structure of the membrane and its physiological function should be investigated as far as possible.

The electrical properties of the membrane will for several reasons be considered in terms of an equivalent circuit. Probably the most important of these is that the measurements have for the most part been made by a direct comparison of the nerve with electrical circuit elements. However, information in this form is quite usable because the techniques for analyzing the behavior of circuits are versatile and powerful and have been widely used. The numerical values and the configuration of the circuit elements may then be used to describe molecular structure on the one hand and physiological function on the other. But it must be emphasized again that any particular circuit is not necessarily unique and should not be interpreted intuitively (Cole, 1928, 1933 *a*, 1937). To avoid as much complexity as possible at the present time, we shall only consider the simplest circuit, shown in Fig. 1, which has a reasonable correspondence to the known membrane properties (Cole and

Baker, 1941 *b*). This circuit makes many concessions to simplicity and several of the obvious defects will be mentioned later (p. 48). Before turning to the physiological applications of the circuit, we shall consider the physical characteristics and implications of the circuit elements themselves.

### MEMBRANE STRUCTURE

#### *Capacity and Resistance*

The membrane capacity,  $C$  (Fig. 1) of the squid axon is about  $1.1 \mu\text{f}/\text{cm}^2$  and it has a constant phase angle of about  $75^\circ$ . These values are in the range of those found for a variety of other cells, and their significance has been discussed recently (Cole, 1940). From physiological evidence, this capacity is probably a characteristic of the ion impermeable portion of the membrane and the phase angle a measure of the dielectric loss in it. For physical reasons, it was suggested that the membrane is a highly organized and coherent structure

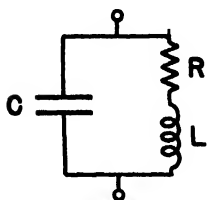


FIG. 1. An approximate equivalent circuit for the membrane of the squid giant axon with capacity,  $C$ , inductance,  $L$ , and resistance,  $R$ .

having a high dielectric constant and a considerable thickness on a molecular scale.

The membrane resistance,  $R$  (Fig. 1) of the squid axon is quite variable, but it is usually of the order of a few hundred ohm  $\text{cm}^2$  for small currents, and similar values have been found for several other cells. This resistance is assumed to be a measure of the ion permeability on the basis of physiological and physical evidence recently summarized (Cole, 1940).

#### *Rectification*

However, as the current is increased, it is no longer found that the resistance,  $R$ , may be considered as a linear circuit element. Measurements of impedance (Cole and Baker, 1941 *a*) and membrane potential (Cole and Curtis, 1941) show that the membrane is a rectifier with a limiting resistance for an outward current flow about one-hundredth of that for an inward flow.

It is very instructive to compare the rectification characteristic of the membrane with those of widely used commercial barrier layer rectifiers, such as copper-copper oxide and selenium. It is found for Cu-CuO that the limiting resistance in forward direction ranges from one-hundredth to one-thousandth of that in reverse direction and for selenium that this ratio is about one-

hundredth. Thus the ratio of one-hundredth found for the axon membrane is quite comparable to that for these rectifiers. However, the similarity probably ends at this point. Although there has been considerable uncertainty as to the exact mechanism of the barrier layer rectifier action, there seems to be little doubt that it is an electronic phenomenon. It seems rather improbable at the present time that there is an electronic conduction in the cell membrane, and we are much more inclined to look for an explanation in terms of an ionic conduction. Such an example has been found by Blinks (1930) in a dried collodion membrane separating different electrolytes, where the rectification was accounted for by the mobility of the permeating ion forced into the membrane by the applied field.

One might also consider the possibility of a conduction by potassium ions alone. It seems reasonable to suppose in such a case that both the concentration and the mobility of this ion are quite small in the membrane structure itself. If the concentration is governed by partition coefficients at the two interfaces we may expect a concentration gradient within the membrane comparable to the external concentration gradient. When there is an inward current flow, a steady state will be set up in which the initial low outside concentration of potassium ions will be decreased by the ions crossing into the membrane. At the inside the potassium ions are readily taken up as they emerge from the membrane and the concentration at each point in the membrane will be reduced. The overall effect will then be an effective increase of the membrane resistance. For outward current flow, the large supply of these ions available at the inner face of the membrane is augmented by the current flow, and at the outer face only a relatively low equilibrium concentration of the ions is possible. As a consequence of this large supply and small delivery the concentration may be expected to increase at every point and so to decrease the resistance.

This picture has not been put into quantitative terms, and it may be simpler to consider it in terms of potential barriers at each interface which are then alternative expressions of the partition coefficients. One may then anticipate a resistance-current relationship of the form  $R = R_0 + R_1 \tanh i/i_0$  and the observed rectification curves are of this general form. However, it seems premature to compare the experimental data with such an analytical expression until each has established on a better basis than at present. It is obvious, however, that the rectification characteristics will at least be an important guide in the formulation and verification of the nature of the mechanism.

### *Inductance*

The idea of a membrane capacity is easy to rationalize and it has not been particularly surprising to find that the axon membrane acts as a rectifier, although the efficiency may be rather unexpected. It is quite another matter

to find that the membrane has the electrical characteristics of an inductance. In ordinary experience an inductance is a characteristic associated only with the storage of energy in a magnetic field, and it is singularly difficult to imagine a membrane structure which would allow an electromagnetic field corresponding to more than a few microhenries. This could be ignored but the actual membrane inductance of one-fifth henry for a  $\text{cm}^2$  cannot.

Although the concept of inductance was originated to express electromagnetic phenomena and our standards of inductance have long been electromagnetic, it is not necessary to restrict the use of the term to this field. An inductance may be defined by means of the relation of the current,  $I$ , and the voltage,  $V$ , in a manner analogous to the operational definition of a resistance,  $R$ , by Ohm's law,  $V = RI$ . Then anything in which it is found that the potential difference is proportional to the rate of change of current is an inductance, electrically speaking, and it is measured as the factor of proportionality,  $L$ , in  $V = L dI/dt$ . Similarly, the definition of capacity,  $C$ , is  $I = C dV/dt$ . Turning to the energy, we find that  $T = 1/2 LI^2$  for the inductance,  $U = 1/2 CV^2$  for the capacity, and the rate of dissipation  $F = RI^2$  for a resistance, completely irrespective of the ultimate mechanisms by which the storage or dissipation of energy may take place.

However, to point out that an inductance can be defined without reference to a magnetic field is hardly a satisfactory explanation of the very large membrane inductance. And indeed it will not be possible to give a complete explanation except in terms of the membrane structure. On the other hand, it may be possible to make the existence of a membrane inductance seem less unreasonable by examples of purely physical structures having inductive characteristics without a corresponding magnetic field.

One example is the carbon filament incandescent lamp. In this instance the explanation of the inductance is relatively simple, and is easily understood by a consideration of the current flow after the application of a constant voltage to the lamp. At the first instant a current will flow corresponding to the cold resistance of the filament. The filament then begins to heat as a result of the current flow at a rate depending upon its heat capacity and to an extent determined by the heat losses. However, a carbon filament has a negative temperature coefficient of resistance and consequently the resistance becomes less than it was initially. Since a constant potential was applied the current rises from the initial value as an exponential function of time and finally reaches a constant value. These electrical characteristics are, however, identical with those of a resistance and inductance network, and it is possible to calculate the thermal characteristics of the lamp from the electrically equivalent structure. When measured in a Wheatstone bridge it is found that such a lamp has an inductive reactance at all frequencies and that the equivalent network of a 60 watt lamp requires an inductance of 30 henries. A much more dramatic

example of an inductance of thermal origin is found in the recently developed Western Electric 1-A Thermistor (Pearson, 1940). In this device a bead of uranium oxide is mounted on two platinum wires and sealed in vacuum. This material also has a negative temperature coefficient which with the heat capacity and losses gives the Thermistor an inductance of many henries. It is not impossible of course that the cell membrane has similar characteristics, but this particular inductance mechanism probably has little value to us except as an example of a non-magnetic inductance.

There is another and more common class of inductances arising from mechanical motions, and the most familiar example of these is the piezoelectric crystal, such as quartz or Rochelle salt. A potential difference applied to two electrodes on one of these crystals tends to change its shape, and conversely if its shape is changed mechanically a potential difference will appear between the electrodes. When the crystal is not constrained, its distortion after the application of a constant potential to the electrodes depends upon the mechanical characteristics of mass, elasticity and damping. As this distortion takes place the electrical charge generated modifies the current flow from the outside circuit in just the same manner as an electrical circuit containing resistance, capacity, and inductance. In this electro-mechanical system the inductance is determined by the mass of the crystal, the capacity by its elasticity, and the resistance by the internal and external frictional losses. When the alternating current has a frequency about that of a natural mechanical frequency of the crystal, the mechanical vibrations may have large amplitude and the electrical reaction on the circuit is particularly powerful. The crystal may then control the frequency of the associated circuit with a high degree of accuracy and serve as a standard of frequency and time. A common type of quartz crystal about 1 cm. square and 1 mm. thick with a natural frequency of 2 megacycles is found to have an inductance of about one-tenth henry or approximately half the inductance of a similar area of axon membrane.

It may seem quite unreasonable to suppose that the axon membrane may be piezoelectric with a natural frequency of a few hundred cycles, but in the present state of our information this possibility cannot be excluded. There is a potential difference across the membrane and probably a very high electrical field strength giving rise to considerable mechanical forces. Any alteration of the field strength by an externally applied potential may be expected to alter the mechanical stress. A resultant deformation reacting on the membrane potential difference might then be measured electrically. However, for a crystal as thin as the axon membrane presumably is to have a natural frequency of a few hundred cycles requires a very high density and very small coefficient of elasticity. But this may not be impossible if the membrane has the laminar structure proposed for the myelin sheath on the basis of x-ray evidence (Schmitt, 1936). This picture of parallel lipo-protein sheets separated by



aqueous layers is highly suggestive of the colloid coacervates such as bentonite, in which the micro-structure, as in the tobacco mosaic virus, is maintained by the interaction of the ion atmospheres (Langmuir, 1938). It is possible that the forces holding a particular particle in its equilibrium position may be small compared to the mass of the particle, and the natural frequency of oscillation about the equilibrium may be of the order required by the membrane measurements. There is of course no direct evidence to support this suggestion, but observations on the double refraction of bentonite sols in an alternating current field (Mueller, 1939; Norton, 1939) are at least helpful. Here it is found that the sign of the double refraction at a frequency of the applied field above about 200 cycles is opposite to that obtained at lower frequencies. We may then expect that impedance measurements of these sols will also show a change of sign of their reactance in this same frequency range although the measurements may be somewhat obscured by surface conductance effects.

If we consider structures containing sources of energy, the possible interpretations of an inductive reactance are almost unlimited. In general we can only say with certainty that the membrane structure is such that it gives rise to a potential difference during current flow which is proportional to the rate of change of the current.

In this discussion of the possible sources of inductance it has been emphasized that the common association of an inductance with a magnetic field may be misleading, and it is equally apparent that the common association of a capacity with a dielectric may be equally misleading. There has been little reason to question the applicability of this concept because it gave so satisfactory a picture of a thin membrane. It has already been mentioned that the elasticity of a piezoelectric crystal gives rise to a capacity. This capacity element is separate and distinct from the dielectric capacity of the crystal which may be obtained when the crystal is constrained and cannot react mechanically to the electric field. Also we find that there are capacities of a thermal origin and as may be expected they arise from a positive temperature coefficient of resistance. A 3 watt tungsten lamp, for example, has been found to have a capacity of about one thousand microfarads. As a consequence we must also be prepared to discard the simple and obvious interpretation of the membrane capacity measurements.

Until there is more complete evidence as to the nature of the membrane structure and function we have no reason to assume that these physical measurements must be explained in terms of physical structures analogous to those which have been used for illustrative purposes. On the contrary, it is probably safest to consider the membrane as far as possible from a physical-chemical viewpoint in which no sharp division can be made between physical and chemical phenomena as has often been done. For example, let us assume that the

variables of the general two factor formulations of excitation (p. 43) can actually be identified chemically, as ions for example, and that the changes of the difference in these ion concentrations correspond to changes of the membrane potential difference. As will be shown, this is electrically equivalent to an inductance, capacity, and resistance, in equations (7) and (8), and the measurements of these elements then constitute an analysis of the chemical kinetics of the membrane system. If we wish to describe the membrane phenomena in purely chemical terms, as is perfectly reasonable and possible, it then seems quite probable that the electrical measurements as they stand contain by far the most accurate and detailed chemical information available at the present time for the axon membrane.

#### MEMBRANE RESPONSE

We shall turn now to the performance of the membrane as a whole, both by itself and in relation to the rest of the axon. It is convenient to divide the field into the two regions of (1) passive or subthreshold phenomena, and (2) active or threshold phenomena. These regions probably should be separated according to whether or not potential energy is released, but for the present the terms will be used without careful definition.

#### *Subthreshold Potential*

The subthreshold phenomena will be considered first in terms of calculated and measured electrical potentials. When a current flows through any part of an axon having an appreciable resistance or impedance, it is obvious that there will be a change of potential. These potentials may be roughly classified as linear and non-linear. For sufficiently small currents, the potential difference is proportional to the current and the factor of proportionality is the resistance for direct current and the impedance for alternating current. When the alternating current characteristics are known it then becomes possible to calculate the linear subthreshold potential difference for the application of any small current. The equivalent circuit of Fig. 1 is, however, an approximate representation of the alternating current characteristics of the membrane and may be used directly.

For a square centimeter of membrane, the capacity,  $C$ , in this circuit is about 1 microfarad, and for simplicity the dielectric loss will be ignored. The resistance,  $R$ , may have a resting value in the neighborhood of 300 ohms per  $\text{cm}^2$  for small currents, and the inductance,  $L$ , is about one-fifth henry. If now  $v_m$  is the potential difference across the membrane at one point and  $i_m$  is the current density at this point, the relation between these two quantities at any time is given by the ordinary differential equation:

$$LC \frac{d^2 v_m}{dt^2} + RC \frac{dv_m}{dt} + v_m = L \frac{di_m}{dt} + Ri_m. \quad (1)$$

This is a familiar second order differential equation with constant coefficients and the methods of solution are well known and direct. However, as the current is increased beyond perhaps 10 per cent of rheobase, it has been found that the resistance,  $R$ , is a rectifier element having a lower resistance for an outward flow of current and a higher resistance for inward flow. This property may be expressed in the form  $v/i = R(i)$  where  $v$  is the potential difference across and  $i$  the current through the element, and we have:

$$LC \frac{\partial^2 v_m}{\partial t^2} + R(i)C \frac{\partial v_m}{\partial t} + v_m = L \frac{\partial i_m}{\partial t} + R(i)i_m, \quad (2)$$

for the non-linear subthreshold potential. The solution of this equation with the variable coefficients has been found for some analytical forms of  $R(i)$ , but the solution must be obtained by numerical, graphical, or mechanical integration when  $R(i)$  is given in numerical or graphical form only. If we turn now from the consideration of a single point on the membrane to a treatment of the axon as a whole we must allow the potential and current to vary along the length of the axon as well as with the time. Equation (2) is then to be replaced by the partial differential equation:

$$LC \frac{\partial^2 v_m}{\partial x^2} + R(i)C \frac{\partial v_m}{\partial t} + v_m = L \frac{\partial i_m}{\partial t} + R(i)i_m. \quad (3)$$

As yet there has been no indication that the exterior and interior of an axon are anything but electrolytic conductors and these may be expressed as the resistances  $r_1$  and  $r_2$  for a unit length of axon. Then with the ordinary assumptions of a negligible radial potential difference inside and outside the membrane and a negligible longitudinal current flow in the membrane we obtain:

$$\frac{\partial^2 v_m}{\partial x^2} = (r_1 + r_2)i_m, \quad (4)$$

where  $i_m$  is now the current per unit length. This becomes the usual cable equation if the membrane is a pure resistance,  $r_m$  for a unit length, and the relation between  $v_m$  and  $i_m$  is given by  $v_m = r_m i_m$ . In the present case, however, this relation is given by equation (3) and the axon behavior is completely determined by the two equations, (3) and (4). In general the membrane current,  $i_m$ , cannot be measured directly under any particular experimental conditions and only the externally applied current,  $I_0(t)$ , is known. The part of this current which crosses the membrane,  $I(t)$ , aptly termed the "penetrating fraction" by Rushton (1937 b), is readily found and we have the further relation

$$I(t) = \int i_m(x, t) dx \quad (5)$$

to be satisfied for each electrode. In the case of no applied current,  $I(t) = 0$ . The behavior of the nerve model under any set of conditions is then to be found by solving the three equations (3), (4), and (5). The complete solution of these partial differential equations is a difficult and laborious procedure which has not yet been carried out for even a single problem, and there are at least as many possible problems as there have been experiments on nerve. The effect of the presence of inductance and rectification elements in the membrane may, however, be illustrated by a few special cases and approximate calculations.

### *Constant Current*

When a small constant current is applied uniformly to a unit area of membrane, the subthreshold potential may be calculated by equation (1) or from the circuit of Fig. 1. This potential will be over-damped, critically damped, or under-damped and oscillatory according as the resistance  $R$  is greater than, equal to, or less than  $2\sqrt{L/C}$ . Also the potential will have the same form for both directions of current flow. For larger currents, the non-linearity of the resistance,  $R$ , must be taken into account. It is obvious that under an anode where the membrane resistance is increased, the circuit will be more and more highly damped as the current is made larger and that the damping will be progressively decreased under the cathode. A rigorous calculation of these potentials from equations (3), (4), and (5) has not been attempted, but the graphical integrations of equation (2) have been carried out assuming a uniform density, as shown in Fig. 2. To compensate in some degree for not solving the partial differential equations, the apparent rectification curve for the applied current (Fig. 4, Cole and Curtis, 1941) was used rather than the otherwise more appropriate curve for a uniform current density (Fig. 5 of that paper).

The curves of Fig. 2 exhibit the same characteristics as the squid axon membrane potentials (Cole and Curtis, 1941) and *Sepia* axon potentials (Arvanitaki, 1939). During current flow at the anode, the damping and consequently the effective time constant during the rising phase of the potentials increase with increasing current, and the final steady levels of potential increase more rapidly than the applied currents. The cathode potentials show progressively less damping until they are under-damped and oscillatory with the steady levels of potential rising less rapidly than the applied currents. The qualitative agreement between these rather crude calculations and the experimental results is sufficiently good to be considered as additional evidence that the electrical picture of the membrane given in Fig. 1 is essentially correct. It is certainly indicated that the *Sepia* axon membrane is a rectifier. In view of the direct evidence for the squid axon and the similarity of the behavior of the *Sepia* it is difficult to believe that the *Sepia* membrane does not have a similar inductance element. There is, however, a discrepancy in the frequency

of the oscillations. For a zero membrane resistance the potentials will have a maximum frequency given by  $\nu_0 = 1/2\pi \sqrt{LC}$ . Using the values of one microfarad/cm.<sup>2</sup> obtained from the transverse impedance measurements and the one-fifth henry cm.<sup>2</sup> from the longitudinal measurements, we find a frequency,  $\nu_0 = 330$  cycles/second. The *Sepia* data give somewhat lower frequencies, as would be expected because of the damping, but the data of Fig. 2 (Cole and Curtis, 1941) give nearly 500 cycles. Until the reasons for this difference are clear or until all of the necessary measurements can be made on a single axon, it will be assumed that this lack of agreement is within the range of variability of the axons and their properties under experimental conditions.

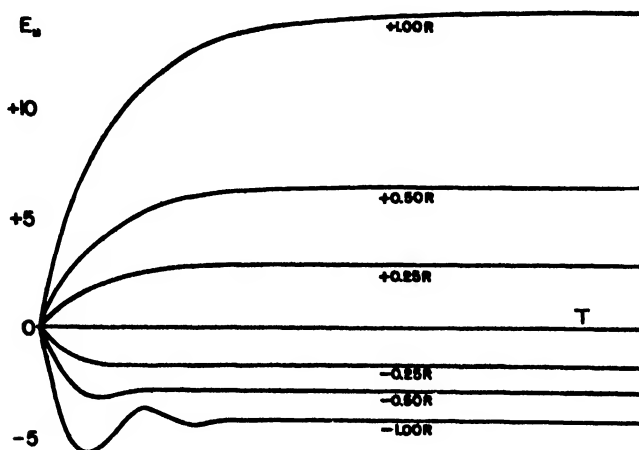


FIG. 2. Approximate graphical calculation of subthreshold potentials from measured membrane characteristics. Changes of membrane potential,  $E_m$  at anode and cathode, vs. time,  $T$ , after the application of constant currents of 0.25, 0.5, and 1.0 rheobase. Increase of potential at anode is upward.

The most striking oscillations of potential have been obtained by Arvanitaki (1939) in partially decalcified preparations. With decreasing calcium, the damping with an applied current is decreased. In the extreme case the damping has become zero or slightly negative, with no applied current. The oscillations of potential are then spontaneous and they increase in amplitude until excitation occurs. It will be particularly interesting to know whether the entire form of the rectification curve of the membrane is changed or only the point on the curve corresponding to zero current flow is altered under these conditions.

#### Short Pulse

This formulation of the electrical properties of the membrane should be equally applicable to the measurements of Hodgkin (1938) on the subthreshold

potential changes in the isolated single crab axon with the application of a short shock, and those of Pumphrey, Schmitt and Young (1940) on the squid axon. However this is apparently not the case since the approximate integration of equation (2) used above does not give the characteristic responses found by Hodgkin. This is evidence that the equivalent circuit of Fig. 1 is not adequate for this preparation and experiment. If the circuit also fails to represent the behavior of the squid axon after a short shock, it follows immediately that the actual and calculated membrane potentials do not agree during the initial phases of the application of a constant current. However, as has been pointed out, the amplifier characteristics have obscured the actual behavior of the membrane potential at short times (Cole and Curtis, 1941), and this prediction cannot be verified from the present records. But Hodgkin's records make it rather probable that the simplifications of either the circuit, or the calculations, or both, are not always justified.

### *Electrotonic Potential*

Suppose that an axon is in a moist chamber, or in a medium restricted by a close-fitting insulating trough, with two very narrow electrodes applied some distance apart. At the first instant that a direct current is applied, the entire membrane current will flow through the capacity because the rectifier and particularly the inductance very effectively oppose a sudden change of current in that branch. The membrane current density at the first instant, although theoretically infinite under an electrode will decrease symmetrically on each side of the electrode and the distribution at the other electrode will be identical except for sign. As time goes on, the current is increasingly transferred from the condenser to the inductance-rectifier branch until, when a steady state is reached, the current is entirely governed by the rectifier. The current flow near the anode is entirely inward and consequently the membrane resistance is increased in this region. Since the current and voltage are no longer changing with time, equation (3) reduces to  $v_m = R(i_m)i_m$ , and now we may calculate the membrane current density at different distances from each electrode by the data of Fig. 4 (Cole and Curtis, 1941) in a manner analogous to that used in determining the membrane potential as a function of the current density. This leads to the results shown in Fig. 3 for three values of anode and cathode current. Quite apart from the analysis, the underlying development of these current distributions is easily seen qualitatively. A small current from the anode increases the membrane resistance immediately under the electrode and so tends to divert the current from this region. As the current is increased it must spread farther along the axon before it can penetrate with reasonable ease. At the cathode the situation is reversed, because the decreased resistance under the electrode reduces the necessity for the current spread that would otherwise take place, and the current concentration becomes relatively larger the higher the current. Then the characteristic length,  $\lambda = \sqrt{R/(r_1 + r_2)}$

may not be a fixed and invariable property of any axon in a steady state as has usually been assumed in theories of excitation and propagation.

It has been known since the time of Pflüger that anelectrotonus had a somewhat wider spatial distribution than cataelectrotonus, and if the electrotonus is directly related to the membrane current flow, this is evidence to indicate that other axon membranes also have rectification properties.

### *Propagated Impulse*

Turning now to the propagated impulse, we may reconsider the difficulties in correlating the phenomena during the rising phase. As has been pointed out (Cole and Baker, 1941*a*), the outward current flow during the foot of the action potential should produce an impedance decrease if the common assump-

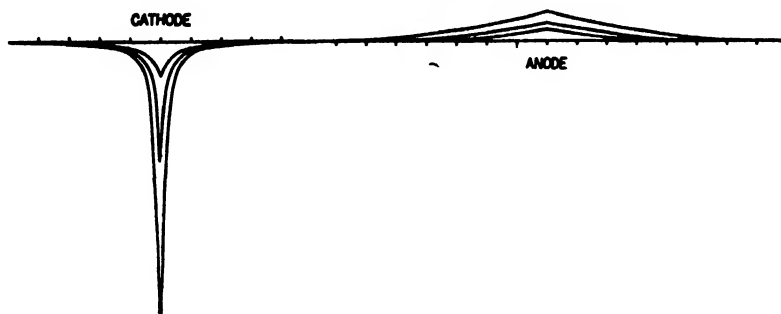


FIG. 3. Steady state membrane current density, ordinate, for narrow electrodes and currents of 0.25, 0.5, and 1.0 rheobase, calculated from measured membrane characteristics. The unit of distance, abscissa, is the characteristic length for small current.

tion of an increased ion permeability of a membrane under a cathode is valid. However, the impedance change was negligible before the point of inflection (Cole and Curtis, 1939) although the steady state effect of current flow was as predicted (Cole and Baker, 1941*a*). The contradiction then remains on the basis of a capacity-resistance membrane structure and the effect of the inductance element should be considered.

The foot of the action potential has been found to be accurately exponential in form in *Nitella* (Cole and Curtis, 1938) and in the squid axon (unpublished experiments), and this form is to be expected, in a capacity or capacity-resistance membrane, ahead of a sudden partial short circuit of the membrane moving with a constant velocity along the axon (Cole and Curtis, 1938). We may now extend this calculation to include any form of membrane impedance so long as it is linear; *i.e.*, if the effect of the rectification may be ignored. Thus the observed exponential form agrees with the impedance observations in requiring that the membrane rectification is not involved in the foot of the

action potential. But this requirement can only be met if both the potential difference across, and the current flow through, the rectifier element are so small that its non-linear characteristics are negligible. Subject to verification, we may assume the resistance of Fig. 1 to be linear and to have a value of 300 ohm cm.<sup>2</sup> and calculate the potential and current during the foot of the action potential. For the membrane capacity, we have the current  $I_o = C dV/dt$ , and if  $V = V_0 e^{t/\tau}$  during this period,  $I_o = C\dot{V}/\tau$  where  $\tau$  is the time constant of the action potential foot. For the inductance-resistance arm of the circuit,  $V = RI_R + L dI_R/dt$ , from which  $I_R = V/(R + L/\tau)$ . Then when  $\tau = 10^{-4}$  sec.,  $\tau/C = 100$  ohms and  $L/\tau = 2000$  ohms. Thus we see that only 5 per cent of the total current flows through the resistance and inductance arm and that only about 15 per cent of the action potential is across the resistance. If the action potential is 50 mv. and the point of inflection is at 25 mv., the capacity current at this time will be  $250 \mu$  amp./cm.<sup>2</sup> The current density through the rectifier will be about  $12 \mu$  amp./cm.<sup>2</sup> at this time and the potential across it will be about 3.6 mv. From Fig. 5 (Cole and Curtis, 1941) it is seen that these values of current and potential difference lie so near the origin that the potential difference is practically proportional to the current. Since the membrane has not entered the non-linear range it may be considered as a simple resistance. With the current flow in this arm and potential difference across the resistance small, we must conclude that the inductive element effectively removes this arm from participation in the foot of the action potential. For slow changes, on the contrary, we may expect the inductance to become less effective and allow a larger potential difference and current flow for the rectifier element. These conclusions find considerable confirmation in unpublished records of the form of the action potential during passage through a polarized region. It has been found that the time constant of the foot of the potential remains the same for anodic and cathodic current flow as the unpolarized axon. Also, the recovery of polarized nerve is slower under the anode and faster under the cathode than normal, but the effect of change of velocity of propagation has not been investigated.

After the application of a constant current, it would be expected that the paths of the current would vary in a similar manner. The current flow through the inductance and rectifier should be negligible at the start and pass through a point of inflection as it builds up to the steady state value. The impedance of the membrane should then show similar characteristics as the current flow modifies the apparent resistance of the rectifier, and a few unpublished records of the impedance change during current flow show the expected characteristics.

Thus we may conclude that, on the basis of the evidence available, there is good reason to believe that the equivalent circuit of Fig. 1 is an adequate correlation and representation of the observed characteristics of the squid axon with the subthreshold potentials at intermediate and long times. At short times



the computations are certainly unsatisfactory, and it is also quite probable that we have here additional evidence that the simple circuit is not a complete representation of the membrane.

### *Threshold and Excitability*

So far, attention has been centered on subthreshold potentials during current flow and the foot of the action potential as measured independent of excitation, and a considerable degree of consistency has been found, along with a number of unsatisfactory aspects. The temptation, however, is very strong to ignore the difficulties for the present and turn to excitation. This work is only a preliminary step in the description of nerve function until it can be extended to include excitation and propagation phenomena. The qualitative description of these phenomena in terms of a membrane breakdown has been so satisfactory that it is certainly not expedient to desert this picture until it can be shown necessary to do so. We may then expect that the two crucial steps in a quantitative description of the phenomena are (1) the conditions for this breakdown to occur, and (2) the nature of the breakdown. But it must be admitted at the present time that there is no satisfactory description of either of these two steps. As a consequence it has been necessary, in order to formulate the initiation and propagation of the nerve impulse mathematically, to make largely unsupported assumptions as to the nature of these two steps. It is possible to assume a variety of membrane circuits and assume that a critical charge, potential difference, or current flow, etc., in any of the elements will result in a change in one or more of the elements of capacity, resistance, inductance and E.M.F. It has been shown that several combinations of these *a priori* possibilities can indeed describe various nerve phenomena rather well, but it seems certain that many other—although perhaps intuitively less satisfactory—permutations of assumptions may do equally well.

We should, therefore, not consider these comparatively specific theories in detail but go back to the experimental data which they attempt, more or less successfully, to correlate. Almost without exception these data have been data of excitation and it appears to have been too optimistic to hope that it would have been possible to determine both the structural and the functional characteristics of nerve from these functional data alone.

These functional data, however, are the backbone of classical electrophysiology of nerve, and they form a self-consistent picture as pointed out qualitatively by Cole (1933 *b*). The quantitative correlations were carried out by Rashevsky, 1933, Monnier, 1934 and Hill, 1936 as two factor theories in which one of the factors,  $U$ , tended towards the excitation and the other,  $V$ , away from it. All the formulations may be considered as special cases of general equations set up by Young (1937)

$$\begin{aligned}\frac{dU}{dt} &= k_{11}U + k_{12}V + a_1I \\ \frac{dV}{dt} &= k_{21}U + k_{22}V + a_2I,\end{aligned}\tag{6}$$

where  $I$  is the stimulating current,  $a_1$  and  $a_2$  are constants, and the  $k$ 's are velocity coefficients, from which the two time constants may be derived. These equations have been considered from a general conventional mathematical viewpoint by Young, but we may consider them in a more informal manner since it has usually been assumed that excitation is governed only by the difference  $U - V = \theta$ , which we may call the excitability.

The two simultaneous first order equations (6) may now be replaced by a single second order equation (Forsythe, 1921<sup>1</sup>) as was mentioned by Rushton (1937<sup>a</sup>) for the form considered by Hill. Then equations (6) may be put in the form (Parrack, 1940)

$$\begin{aligned}\frac{d^2\theta}{dt^2} - (k_{11} + k_{22})\frac{d\theta}{dt} + (k_{11}k_{22} - k_{12}k_{21})\theta \\ = (a_1 - a_2)\frac{dI}{dt} - [(k_{21} + k_{22})a_1 - (k_{12} + k_{11})a_2]I\end{aligned}\tag{7}$$

This equation correlates a vast amount of data as reviewed by Katz (1939) for various forms of electrical stimuli, square pulse, condenser discharge and charge, and alternating current. However, a difficulty arose when an oscillatory excitability was found for a single frog fiber by Erlanger and Blair (1936). This phenomenon was investigated in more detail and particularly in relation to nerve calcium and alternating current threshold by Monnier and Coppée (1939) and it was concluded that one of the two time constants in Monnier's formulation would have to be a complex quantity.

It can be shown quite generally that under certain conditions  $U$  and  $V$  may be oscillatory (Forsythe, 1921<sup>2</sup>), and these conditions have been applied to population problems by Lotka (1925<sup>3</sup>) and repetitive discharge by Householder (1939). Considering only the excitability, it is seen that equation (7) has an oscillatory solution when  $k_{12}$  and  $k_{21}$  are of opposite sign and  $(k_{11} - k_{22})^2 < 4k_{12}k_{21}$ . These are then the conditions for complex time constants in the sense of Monnier and Coppée and in other cases the time constants are real. Thus we see that equation (7) for the excitability of a nerve can represent a very considerable amount of the available data.

<sup>1</sup> Page 350.

<sup>2</sup> Page 342.

<sup>3</sup> Page 78.

If we now may represent the axon membrane by a capacity, inductance, and constant resistance, the relation between the potential difference across it,  $V$ , and the current,  $I$ , given by equation (1), is

$$\frac{d^2 V}{dt^2} + \frac{R}{L} \frac{dV}{dt} + \frac{1}{LC} V = \frac{1}{C} \frac{dI}{dt} + \frac{R}{LC} I. \quad (8)$$

This equation also has an oscillatory solution when  $R < 2\sqrt{L/C}$ , and two real time constants for  $R > 2\sqrt{L/C}$ . The similarity of equations (7) and (8) is highly suggestive, and one is tempted at this point to identify the excitability  $\theta$  with the membrane potential,  $V$ , and combinations of the  $k$  and  $a$  coefficients with  $R$ ,  $L$ , and  $C$ . It is obviously possible to replace the various quantities and coefficients defined only by excitation measurements with constants which are physically defined and measured without reference to excitation. Then all data which are represented by equation (7) may be represented equally well by equation (8) if the numerical values of  $R$ ,  $L$ , and  $C$  will permit.

It has long been a favorite assumption that excitation took place when a threshold change of the membrane potential occurred. Qualitative support for this assumption is seen in the records of the potential following the application of a constant stimulus. As the threshold is reached the action potential appears to begin at the time of the first minimum of the subthreshold potential (Cole and Curtis, 1941). The recent investigation by Pumphrey, Schmitt, and Young (1940) of the excitability and local potential following short shocks also indicates a close correlation between these two quantities. Consequently we are entirely justified in assuming a close relation between the two, but for the present it is not certain that a simple proportionality applies for all types of stimulation. Furthermore in repetitive response to a constant stimulus the successive excitations take place at approximately the times when the successive maxima of the subthreshold potential would be expected (Arvanitaki, 1939). However, sufficient data are not available in such experiments to satisfactorily define the time at which excitation takes place and determine the membrane potential at that point.

On the other hand, in the propagated impulse it does seem very plausible that excitation takes place at about the point of inflection of the rising phase of the action potential, and it has often been assumed that a threshold change of membrane potential has occurred at this point. From unpublished records of the membrane potential during the passage of an impulse through a polarized region there is evidence that the potential at the point of inflection depends upon the polarizing current and also that the difference between this potential and the steady state potential depends upon the current. In other words, it has not yet been possible to find any membrane potential or change of membrane potential having a value at the point of inflection which is independent of the polarizing current.

Although equation (8) may be solved for all possible forms of stimulating current, the general features are most easily seen for alternating current stimulation. The current necessary for a constant value of the potential difference may be computed directly as a function of frequency from equation (8). It is also proportional to the reciprocal of the absolute value of the impedance of the equivalent circuit of Fig. 1. The membrane impedance has a maximum for all values of the damping,  $\eta = R\sqrt{C/L}$ , less than 0.7 and at a frequency below the natural undamped frequency  $1/2\pi\sqrt{LC}$  as seen in Figs. 3 b, 5, and 7 of the previous paper (Cole and Baker, 1941 b). An optimum frequency for excitation is then to be expected somewhat below 330 cycles for the squid axon membrane. Adequate data are not available, but preliminary results between 150 cycles and 200 cycles at least indicate that the orders of magnitude of the membrane capacity,  $C$ , and inductance,  $L$ , are correct.

The excitability oscillations at a frequency of 200 cycles (Erlanger and Blair, 1936; Monnier and Coppée, 1939) and optimum frequency for alternating current excitation from 100 to 250 cycles (Katz, 1939; Monnier and Coppée, 1939) for the frog sciatic nerve are too near the region of the undamped natural frequency of the squid membrane to be ignored. This approximate agreement may certainly be taken as an indication that the electrical structure of the two nerves may be similar.

It has usually been found that the threshold alternating current stimulus, as a function of the logarithm of the frequency, was symmetrical with respect to the optimum frequency. This characteristic is dependent upon the presence of complete accommodation, which means that the coefficients of the current,  $I$ , in equations (7) and (8) must be zero (Parrack, 1940). Since no evidence of incomplete accommodation was found in the two squid axons tested, it is again apparent that the simple circuit of Fig. 1 is not adequate on the basis of the present assumptions.

However interesting as this comparison may be, it cannot be taken too seriously. From a rigorous point of view it probably must be regarded at present as coincidental and fortuitous. In the first place the excitability equation (7) takes into account distributed effects and the variation of membrane current flow at and near the electrodes, while the potential equation (8) applies only to a uniform current density. In the second place, the potential equation (8) does not take into account the rectifier characteristic of the membrane. Thirdly, a variety of observations indicate that a liminal change of membrane potential is not the necessary and sufficient condition for excitation. When it becomes possible to make calculations from equations (4), (5), and (6) and more complete information on excitation is available, we shall be in a better position to judge the extent to which the physical data may explain the phenomenological formulation of excitability.

It is interesting to note that the two factor theories have been created for the sole purpose of explaining the behavior of nerve at relatively long times as compared with chronaxie or excitation time, and that a linear relation between cause and effect has been assumed here as well as for short times. The electrical measurements, however, indicate a non-linear element which probably becomes a controlling factor at long times but is relatively unimportant, as we have seen, at short times. However, the excitability data may not be symmetrical for anode and cathode (Parrack, 1940) although this asymmetry is much less than is found for the membrane potential or calculated by integration of the rectifier equation (2). Mention has already been made of the relative spread of steady state electrotonus at the anode and cathode (p. 39).

As a basis for calculation, Rushton (1937 *b*) and Offner, Weinberg, and Young (1940) have each explicitly assumed a membrane structure, and a condition and nature of excitation which are equivalent. On the basis of these assumptions, Rushton found it necessary to have a minimal length of the axon fulfill this excitation condition before an impulse could be propagated. As soon as it is recognized that there can be a departure from the resting or linear properties of the axon membrane before excitation takes place, the minimal length becomes difficult to specify exactly. It is quite possible that this length is an equivalent of the region over which the requisite non-linear change has taken place without excitation. Similarly, we may expect to find that the non-linear responses of Pumphrey, Schmitt, and Young (1940), which they referred to as physiological, are to be related to the electrical non-linearity of the membrane. A considerable amount of experiment and careful theory, taking into account the capacity, rectification, and inductance, will be necessary to verify such suggestions.

Until reasonably detailed calculations have been carried out, it is unsafe to predict the performance of the equivalent circuit for high frequency currents at and near the threshold for excitation. It may, however, be expected, optimistically perhaps, that, as has been suggested, the membrane rectification plays a considerable rôle in these various phenomena (Gildemeister, 1930; Katz, 1937; Rosenbleuth, Reboul, and Grass, 1940). For example, the considerable delay in the appearance of excitation at high frequencies would be explained by a rectification which allowed an average cathode direct current to flow across the membrane under the electrode. This current might be expected to build up at a rate determined largely by the natural frequency and damping of the membrane until the threshold was reached. It is obvious, however, that we cannot expect this characteristic at one electrode and not at the other. Consequently, this postulated average cathode current is a local rather than an external current and there must be nearby anode regions and perhaps even more distant alternating cathode and anode regions. Under some conditions the anode regions may have so high a current density as to give

the alternating current blocks of conduction which have often been observed, and it is then completely consistent to have the well known "break" excitation from this region when the alternating current is stopped.

#### DISCUSSION

In the first place it is interesting to consider the lack of earlier direct evidence for rectification and inductance elements in nerve. If these elements are present in other nerves, the usual small size of the axons is probably the most important obstacle to their measurement. The measurements must be made at low frequencies or long times, relatively speaking, to avoid having the membrane capacity carry too large a fraction of the membrane current. The membrane then presents so high an impedance that the current will flow almost entirely in the extra-cellular fluids unless a large area of membrane is exposed and the alternative paths are reduced to a minimum. These requirements are most easily approached by a large single axon such as Young's squid giant axon preparation. However, transverse impedance measurements on this axon failed to give any indication of an inductance or even a finite membrane resistance because there was so much fluid surrounding the axon that the membrane current at the low frequencies was negligible with the measuring sensitivity available. This difficulty is greatly reduced in the longitudinal measurements because a relatively large proportion of the electrode current crosses the membrane. However, rectification was not found in the investigation of membrane resistance by direct current longitudinal measurements (Cole and Hodgkin, 1939) where one might have expected a change of resistance with current strength. The decrease of resistance at the cathode certainly compensated for the increase at the anode to some extent in these experiments, and the net result of these two effects may be calculated from the data of Fig. 5 (Cole and Curtis, 1941). The total current flow through the membrane is given by  $i_2 = [r_1/(r_1 + r_2)]i_0$ , where  $i_0$  is the electrode current, and  $r_1$  and  $r_2$  are the external and internal resistances per unit length. The apparent resistance of the membrane for one electrode,  $r_m = V_m/i_2$ , where  $V_m$  is the change of membrane potential, is calculated as the function of the cathode current given in Fig. 4. The membrane current flow for the second electrode is in the opposite direction, giving the anode curve. For the two membranes in series we then have the sum of the two individual resistances as shown by the third curve. The change of resistance with current for the membranes alone is about 10 per cent, but when the internal and external resistances are included the maximum variation of the measured resistance predicted for a 1 cm. inter-polar distance is about 1 per cent. Not only is this within the limits of measurement but the currents considered here are much larger than those used by Cole and Hodgkin. Furthermore, in preliminary experiments on the squid axon with one killed end, the variation of the longitudinal direct current re-

sistance with the magnitude and direction of the current has been measured (Guttman, unpublished).

Since the membrane circuit discussed is admittedly inadequate, a few of the more obvious defects may be pointed out. The membrane electromotive force has been assumed constant and is therefore omitted except insofar as it appears in the rectifier element. The capacity, for example, has been adequately demonstrated to have considerable dielectric loss, which is ignored for simplicity. The inductance is treated as independent of frequency but this cannot be verified until the complicated effect of dielectric loss is adequately

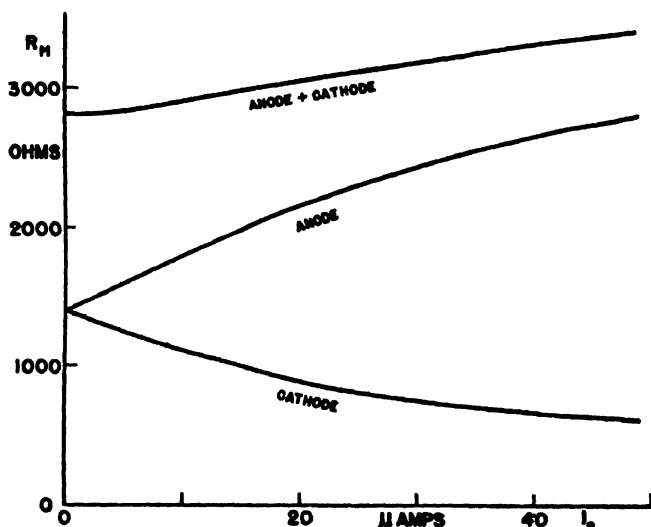


FIG. 4. Effective resistance of axon membrane,  $R_M$ , vs. total measuring current,  $I_0$ , for anode and cathode separately and in series.

calculated. Also, both the capacity and inductance are assumed to be quite invariable. This seems to be justified for the capacity, but there is little evidence as yet concerning the stability of the inductance. Turning to the rectifier, it has been noted that the theoretical impedance loci for the circuit shown in Fig. 1 are only in qualitative agreement with the measurements. A decrease of the resistance should decrease the damping and so increase the inductive reactances at low frequencies. But it was found that the inductive reactance decreased as the direct current resistance of the axon membranes decreased when they deteriorated and lost excitability.

The measurements of the changes of transverse impedance during current flow and the membrane potential during current flow have given similar rectification characteristics for the membrane. The membrane potential was

considered under the direct current steady state conditions in which the inductance would have no effect, but the transverse impedances were measured at frequencies of 2 kilocycles and higher. Under these conditions, the effect of a rectifier placed as indicated in Fig. 1 would be considerably reduced by the inductance and consequently dependent upon the frequency, but the measurements were interpreted on the basis of a frequency independent rectifier element in parallel with the capacity. The addition of such an element in the circuit of Fig. 1 will lessen or alleviate some of these inconsistencies, but more adequate experimental data are needed to specify such an additional factor definitely and justify the increased complexity of analysis.

The corollaries of a piezoelectric membrane structure must not be overlooked. If an applied potential difference can deform the membrane, the action potential should have a similar effect. In either case a change of the membrane double refraction is to be expected, but careful experiments by Schmitt and Schmitt (1940) failed to show such an effect during the passage of an impulse in the squid axon. It is of course possible that the axon membrane is too thin for such observations, but this can only be conjecture at present. On the other hand, if a potential difference can deform the membrane, a deformation can change the potential difference. If there is a threshold potential difference for excitation there should also be a threshold mechanical deformation with similar temporal characteristics. Mechanical excitation of nerve has long been known, but it has been difficult to measure experimentally. None the less the temporal characteristics as exhibited by the strength-duration curve (Blair, 1936) are at least in the same general range as for electrical excitation.

It has already been mentioned that simple calculations of the potential after a short pulse of current applied to the circuit of Fig. 1 do not agree with the observations on crab and squid axons. Also the excitation characteristics to be expected from this circuit do not include the case of complete accommodation which has usually been treated theoretically and investigated experimentally. These defects may well be results of the over-simplification of the membrane properties which has been made. Preliminary measurements and calculations indicate that the modifications of the membrane circuit which are needed to explain the impedance measurements more satisfactorily are in the proper direction to reduce the discrepancies between the observed and predicted potentials and excitabilities.

Measurements of a range of forms have revealed a considerable consistency in the value of the membrane capacity and in a few cases it has been found also that this capacity is quite independent of the physiological function and condition of the cell. The reasonable conclusion is then that the capacity represents a highly constant and inert structure of the membrane. It has been found that a number of under- and over-damped oscillatory nerve phenomena occur



in the range from 100 to 500 cycles and an inductance has been shown to be an essential element in squid axon structure. If this element also appears in frog and *Sepia* axons, the order of magnitude of the inductance will be the same as for the squid, for a variety of conditions and experiments. It would then seem very likely that all of these and probably other nerve phenomena are dependent upon a membrane inductance and that this inductance is at least as constant and inactive as the membrane capacity. It is much too early to predict that an inductance is a characteristic of a nerve membrane, much less of all cell membranes, for it could conceivably be the distinguishing characteristic of membranes having a propagated activity.

#### SUMMARY

Previous measurements have shown that the electrical properties of the squid axon membrane are approximately equivalent to those of a circuit containing a capacity shunted by an inductance and a rectifier in series. Selective ion permeability of a membrane separating two electrolytes may be expected to give rise to the rectification. A quasi-crystalline piezoelectric structure of the membrane is a plausible explanation of the inductance. Some approximate calculations of behavior of an axon with these membrane characteristics have been made. Fair agreement is obtained with the observed constant current subthreshold potential and impedance during the foot of the action potential.

In a simple case a formal analogy is found between the calculated membrane potential and the excitability defined by the two factor formulations of excitation. Several excitation phenomena may then be explained semi-quantitatively by further assuming the excitability proportional to the membrane potential.

Some previous measurements and subthreshold potential and excitability observations are not consistent with the circuit considered and indicate that this circuit is only approximately equivalent to the membrane.

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# THE EFFECT OF CALCIUM AND OTHER IONS ON THE AUTOCATALYTIC FORMATION OF TRYPSIN FROM TRYPSINOGEN

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The effect of salts upon the formation of trypsin from its inactive precursor, trypsinogen, has been extensively studied. Delezenne (1905), Zunz (1906, 1907), Ayrton (1909), Meyer (1910), and Wohlgemuth (1912) showed that the spontaneous activation of fresh pancreatic juice or extracts of fresh pancreas was hastened upon the addition of calcium or other alkaline earth salts, the former being most effective. De Souza (1908) and Mellanby and Woolley (1913) considered that the accelerating action of calcium salts was due to neutralization since the amount of calcium chloride required corresponded closely with that necessary to precipitate the carbonate of the pancreatic juice. They found that barium and strontium chloride or neutralization with hydrochloric acid was equally as effective as calcium chloride. Waldschmidt-Leitz (1924), on the other hand, was unable to show any increase in spontaneous activation with calcium salts. More recently Farber and Wynne (1935) found that the activity of impure pancreatic proteinase was definitely stimulated by calcium salts but thought it possible that this increase in activity was due to the removal of inhibitors. The contradictory results found in the literature are undoubtedly due both to the impure materials and to the diversity of the conditions used. This paper is a summary of an extensive study<sup>1</sup> of the effect of salts on the autocatalytic formation of trypsin from purified crystalline trypsinogen.

Crystalline trypsinogen and trypsin have been isolated from beef pancreas by Kunitz and Northrop (1934, 1936). They found that a solution of crystalline trypsinogen containing a trace of trypsin was gradually changed into trypsin in the pH range of 5.0 to 9.0. The reaction, however, was incomplete.

<sup>1</sup> Dissertation submitted by Margaret R. McDonald to the Graduate Faculty of Rutgers University, June, 1940, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

This was found by Kunitz (1939) to be due to another reaction occurring during the process of formation of trypsin, namely the transformation of part of the trypsinogen into an inert protein which could not be changed into trypsin by any of the known means. The experimental curves for the rate of formation of trypsin as well as for the rate of formation of inert protein were found to be symmetrical S-shaped curves closely resembling those of simple autocatalytic reactions. The kinetics of the formation of trypsin and of inert protein from trypsinogen could be explained quantitatively on the theoretical assumptions that both reactions were of the simple unimolecular type, that in each case the reaction was catalyzed by trypsin, and that the rate of formation of each of the products was proportional to the concentration of trypsin and to the concentration of trypsinogen in solution. During the process of formation of trypsin and inert protein the ratio of the concentrations of the two products in any given reaction mixture remained constant, was equal to the ratio of the velocity constants of the two reactions, and was independent of the original concentration of trypsinogen.

The present studies show that salts influence the transformation of trypsinogen into trypsin in one of the following ways.

(a) Increasing both the rate of formation of trypsin and the rate of formation of inert protein. (b) Decreasing both the rate of formation of trypsin and the rate of formation of inert protein. (c) Increasing the rate of formation of trypsin and decreasing the rate of formation of inert protein. (d) Decreasing the rate of formation of trypsin and increasing the rate of formation of inert protein.

The effect of salts upon the ultimate amounts of trypsin and inert protein formed depends upon the relative influence of the various ions on the rate of both reactions since the final amounts of the products formed depend on the ratio of the velocity constant,  $K_1$ , for the formation of trypsin to the velocity constant,  $K_2$ , for the formation of inert protein. The influence of salts is due to both the cation and the anion; the chemical nature of the ions is more important than their valency.

The behavior of the calcium ion is particularly striking since it inhibits completely the formation of inert protein even in concentrations as low as 0.02 M with the result that the trypsinogen is converted quantitatively into trypsin. In the presence of calcium salts, therefore, the transformation of trypsinogen into trypsin by means of trypsin follows the course of a simple unimolecular autocatalytic reaction.

The ions studied may be arranged in the following approximate series, the first members in each group having an increasing and the last members a decreasing effect.

*A. Effect on the Rate of Formation of Trypsin*

Anions	Cations
Sulfate, citrate, oxalate, tartrate, acetate	Calcium
Fluoride, chloride	Strontium
Bromide	Barium, magnesium
Nitrate	Sodium
Iodide	Lithium
	Potassium, ammonium
	Rubidium
	Caesium

*B. Effect on the Rate of Formation of Inert Protein*

Anions	Cations
Iodide	Barium
Nitrate	Magnesium
Tartrate, citrate, bromide	Lithium
Oxalate, acetate, sulfate	Potassium
Chloride	Sodium
Fluoride	Ammonium, rubidium, caesium
	Strontium
	Calcium

*C. Effect on the Ultimate Percentage of Trypsinogen Changed into Trypsin*

Anions	Cations
Acetate, sulfate, oxalate, citrate, tartrate, fluoride, chloride	Calcium
Bromide	Strontium
Nitrate	Magnesium, sodium
Iodide	Rubidium, ammonium, lithium, potassium
	Caesium, barium

## EXPERIMENTAL

*General Procedures*

The methods used for the study of the formation of trypsin and of inert protein from trypsinogen were those previously described (Kunitz, 1939).<sup>7</sup> Crystalline trypsinogen was prepared from extracts of fresh beef pancreas by the method of Kunitz and Northrop (1936), further purified and freed from inhibitor by precipitation with trichloroacetic acid, and dialyzed against 0.005 M phosphoric acid. Complications due to protein hydrolysis were avoided by keeping all reaction mixtures at 5°C. The solutions were kept sterile by the addition of 0.1 ml. 1 per cent merthiolate in 1.4 per cent borax to each 100 ml. solution. Salts of Merck's reagent grade were employed.

The pH of each activation mixture was adjusted when necessary with phosphoric acid or potassium hydroxide. The measurements were made by means of a low re-

sistance glass electrode of the Mouquin and Garman type (1937). In the range of pH used the salt error was negligible (Gardiner and Sanders, 1937; Cranston and Brown, 1937; Dole and Wiener, 1937). In a comparison of measurements with the glass and hydrogen electrodes the maximum variation in pH observed was 0.04 with an average deviation of 0.02.

#### EXPERIMENTAL RESULTS

##### 1. *Effect of Ammonium Salts on the Autocatalytic Formation of Trypsin.*—

The experimental data for the transformation of trypsinogen into trypsin in various concentrations of ammonium salts are given in Figs. 1 *a*, 1 *b*, 1 *c*, 1 *d*. Fig. 1 *a* shows that not only does the rate of formation of trypsin increase with increasing concentration of ammonium sulfate but that the final amount of trypsin formed is also greater. In the case of ammonium chloride (Fig. 1 *b*) the rate of formation of trypsin decreases with increasing concentration of salt while the final amount of trypsin formed is practically the same. Increasing the concentration of ammonium nitrate (Fig. 1 *c*) decreases both the rate of formation of trypsin and the amount of trypsin formed. The rate of formation of trypsin decreases with increasing concentration of ammonium acetate (Fig. 1 *d*) while the final amount of trypsin formed increases.

The diversity in the results is due to the difference in the effect of the various ammonium salts not only on the velocity constant,  $K_1$ , for the formation of trypsin but also on  $K_2$ , the velocity constant for the formation of inert protein. The initial slopes of the curves are proportional to  $K_1$  while the percentage trypsinogen ultimately changed into trypsin is equal to  $\frac{r}{1+r} \times 100$ , where  $r = \frac{K_1}{K_2}$ . The observed values for the percentage trypsinogen finally changed into trypsin and the calculated values<sup>2</sup> of  $K_1$ ,  $K_2$ , and  $\frac{K_1}{K_2}$  are given in Table I. In the presence of ammonium sulfate both velocity constants at first decrease slightly and then increase rapidly as the concentration of salt increases. The

<sup>2</sup> The velocity constants  $K_1$  and  $K_2$  for the formation of trypsin and inert protein respectively were calculated by means of the equations derived by Kunitz (1939), namely

$$K_1 + K_2 = 2.3 m$$

and

$$K_1 = \frac{2.3 m}{G_0} \times \frac{A_\infty - A_0}{A_\infty}$$

where  $m$  is the slope of the straight line resulting when the values of  $\log \frac{A}{A_\infty - A}$  are plotted against  $t$ .

$G_0$ , the original concentration of trypsinogen

$A_0$ , the original concentration of trypsin

$A$ , the concentration of trypsin at any time  $t$

and  $A_\infty$ , the final concentration of trypsin.

effect is much greater on  $K_1$ , however, so that the ratio  $\frac{K_1}{K_2}$  and hence the final amount of trypsin formed increases with increasing concentration of ammonium sulfate.

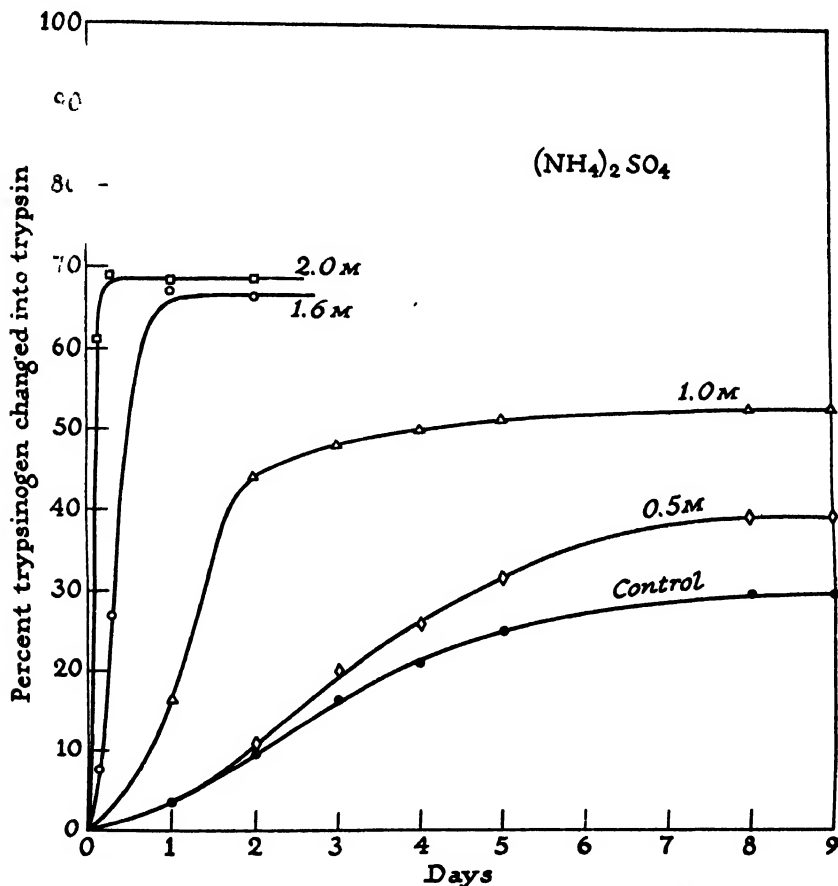


FIG. 1a

FIGS. 1a, 1b, 1c, 1d. Effect of various concentrations of ammonium salts on the formation of trypsin. Activation mixtures: various concentrations of ammonium salts in 0.1 M phosphate buffer pH 7.6 and containing 0.01 mg. trypsinogen protein nitrogen per ml.

The velocity constants,  $K_1$ , and  $K_2$ , decrease with increasing concentration of ammonium chloride, the rate of decrease being the same for both reactions. The value of  $\frac{K_1}{K_2}$  is therefore unchanged and the percentage trypsinogen changed into trypsin is the same regardless of the concentration of ammonium chloride.



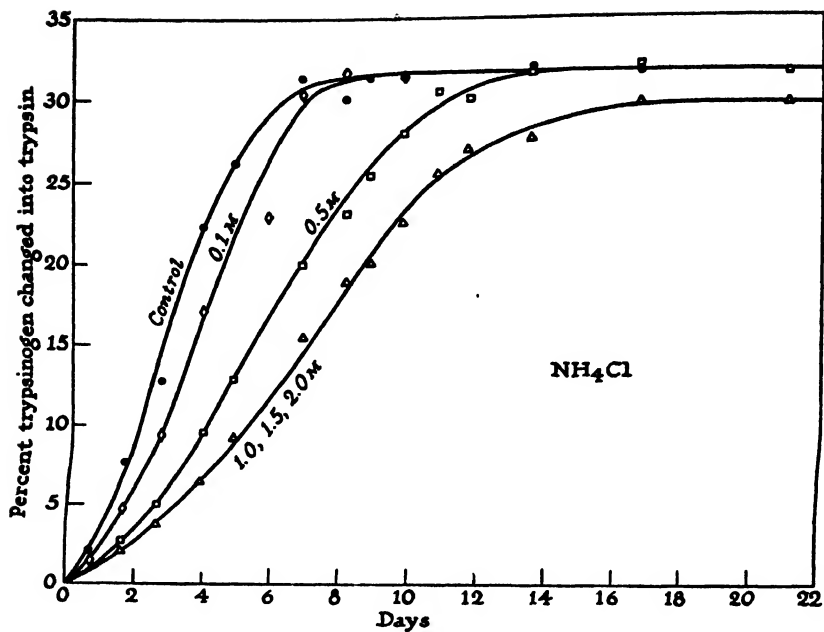


FIG. 1 b

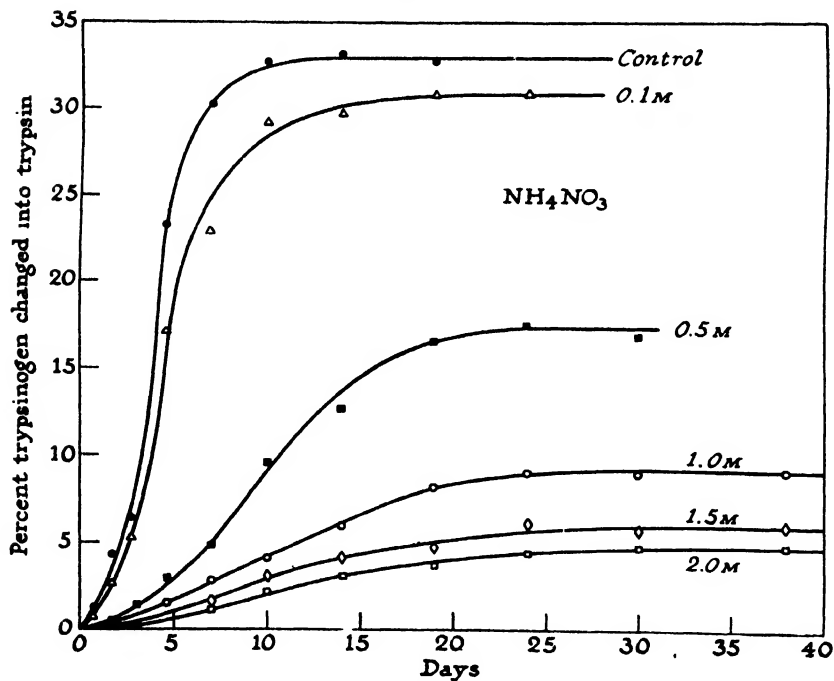


FIG. 1 c

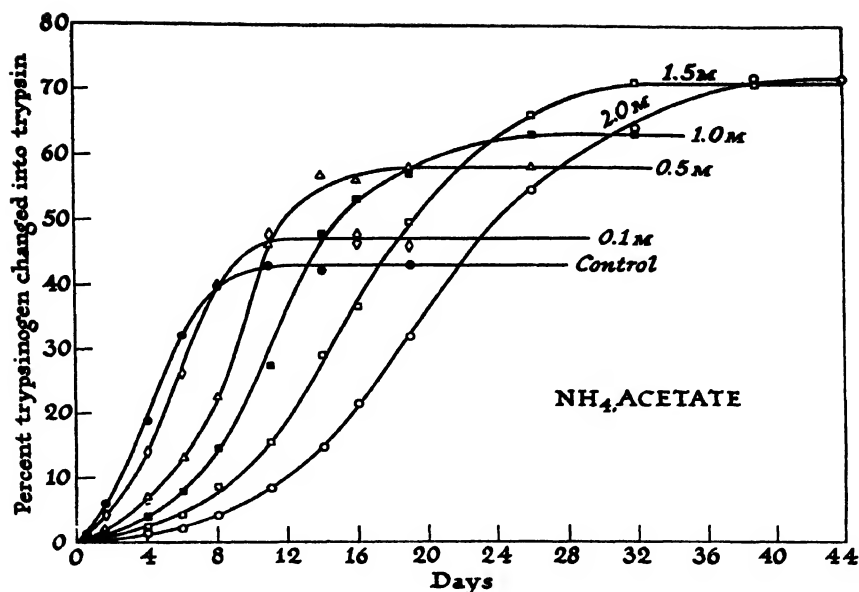


FIG. 1d

TABLE I  
Effect of Ammonium Salts

Molar concentration. ....		0	0.1	0.5	1.0	1.5	2.0
Salt	pH	Per cent trypsinogen changed into trypsin (observed)					
Ammonium sulfate . . . . .	7.3	29	30	39	53	66	68
" chloride. . . . .	7.2	31	31	32	30	30	30
" nitrate. . . . .	7.3	33	31	16	9	6	5
" acetate . . . . .	7.0	42	48	59	63	71	71
		$K_1$ (per mg. P.N. per day)					
" sulfate . . . . .	7.3	89	83	92	235	1450	4850
" chloride. . . . .	7.2	103	85	60	52	52	52
" nitrate. . . . .	7.3	87	63	26	16	13	10
" acetate. . . . .	7.0	83	60	43	32	24	17
		$K_2$ (per mg. P.N. per day)					
" sulfate . . . . .	7.3	225	194	148	219	740	2210
" chloride. . . . .	7.2	230	187	131	120	120	124
" nitrate. . . . .	7.3	177	142	135	153	195	197
" acetate . . . . .	7.0	114	66	33	18	10	7
		$\frac{K_1}{K_2}$					
" sulfate . . . . .	7.3	0.40	0.43	0.62	1.07	1.96	2.19
" chloride. . . . .	7.2	0.45	0.46	0.46	0.43	0.43	0.42
" nitrate. . . . .	7.3	0.49	0.44	0.19	0.10	0.07	0.05
" acetate. . . . .	7.0	0.73	0.91	1.30	1.78	2.40	2.42

The final amount of trypsin formed from trypsinogen in the presence of ammonium nitrate decreases rapidly with increasing concentration of the salt since the velocity constant for the formation of trypsin decreases while the velocity constant for the formation of inert protein remains practically unchanged.

Increasing the concentration of ammonium acetate increases the percentage trypsinogen changed into trypsin by decreasing  $K_2$  to a much greater extent than  $K_1$ .

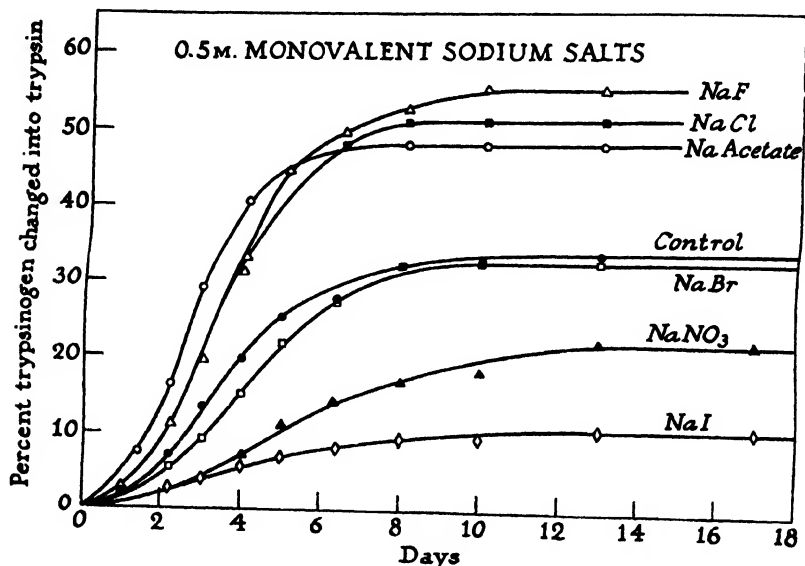


FIG. 2a

FIGS. 2 a, 2 b, 2 c. Effect of various anions on the formation of trypsin. Activation mixtures: various sodium salt solutions in 0.1 M phosphate buffer pH 7.6 and containing 0.01 mg. trypsinogen protein nitrogen per ml. Final pH = 7.1.

*2. Effect of Various Anions on the Autocatalytic Formation of Trypsin.*—The striking differences in the effects of various anions on the transformation of trypsinogen into trypsin and inert protein found with ammonium salts were obtained also with sodium salts, as is illustrated in Figs. 2 a, 2 b, 2 c, and Table II. These show, in general, that the sulfate, citrate, oxalate, tartrate, acetate, fluoride, and chloride ions accelerate the rate of formation of trypsin, the effect decreasing in the order given,<sup>3</sup> while the iodide, nitrate, and bromide ions decrease its rate of formation. The rate of formation of inert protein is accelerated by iodide, nitrate, tartrate, and citrate and decreased by fluoride, chloride, sulfate, acetate, oxalate, and bromide ions. In the presence of

<sup>3</sup> All series in this paper are arranged in order of decreasing effect of the ions.

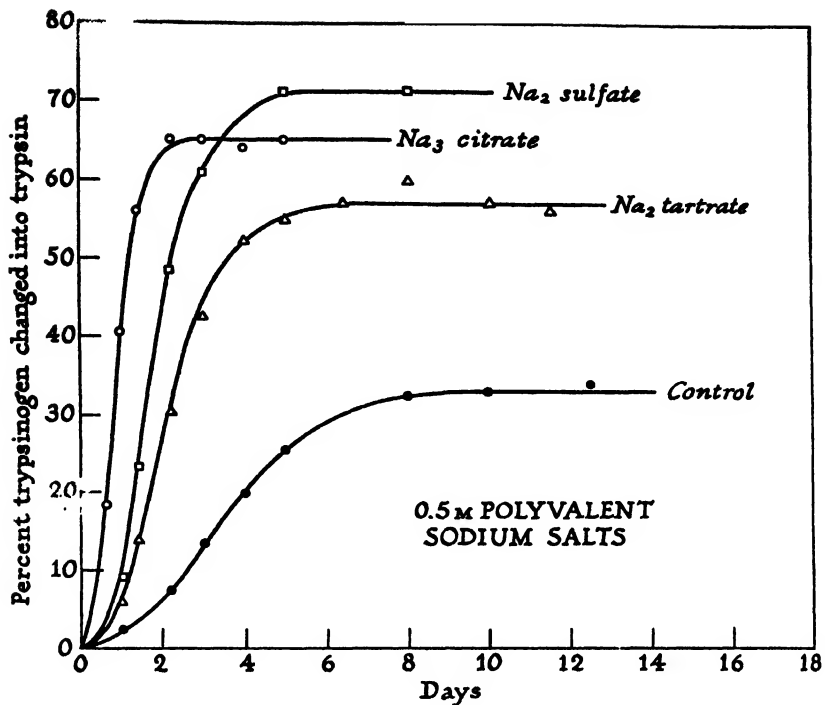


FIG. 2b

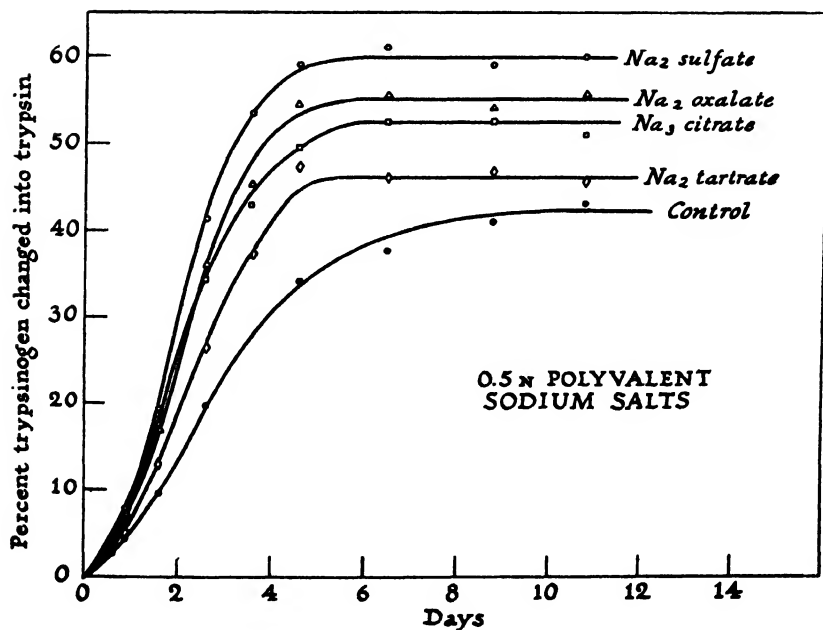


FIG. 2c

sulfate, oxalate, citrate, tartrate, fluoride, chloride, and acetate the ultimate amount of trypsin formed is greater than without the salt while with iodide, nitrate, and bromide less trypsin is formed. Although there is in general a greater tendency for the polyvalent anions to increase the rate of formation of trypsin, the valency of the anion is not the controlling factor since much greater differences were observed between the halides, for example, than between the acetate, citrate, and tartrate.

3. *Effect of Various Cations on the Autocatalytic Formation of Trypsin.*—Figs. 3 *a* and 3 *b*, show the experimental data for the formation of trypsin from trypsinogen under the influence of various salts of the alkali metals. The

TABLE II  
*Effect of Sodium Salts*

Salt	Molar concentration	Per cent trypsinogen changed into trypsin (observed)	$K_1$	$K_2$	$\frac{K_1}{K_2}$
<i>per mg. P.N. per day</i>					
Control.....		33	87	176	0.49
Sodium citrate.....	0.5	65	455	244	1.87
“ sulfate.....	0.5	68	231	107	2.16
“ tartrate.....	0.5	57	214	161	1.33
“ acetate.....	0.5	48	131	139	0.94
“ fluoride.....	0.5	55	104	84	1.24
“ chloride.....	0.5	51	104	100	1.04
“ bromide.....	0.5	32	81	171	0.47
“ nitrate.....	0.5	22	59	203	0.29
“ iodide.....	0.5	11	47	380	0.12
Control.....		42	92	125	0.74
Sodium sulfate.....	0.25	60	141	93	1.52
“ citrate.....	0.17	52	135	122	1.11
“ oxalate.....	0.25	56	131	102	1.29
“ tartrate.....	0.25	46	129	140	0.92

calculated values of  $K_1$  and  $K_2$  are given in Table III. The salts of sodium and lithium were found to increase the rate of formation of trypsin while those of caesium, rubidium, and potassium were found to decrease it. The rate of formation of inert protein was decreased by the salts of rubidium, caesium, sodium, potassium, and lithium, the latter having the least effect. The largest ultimate amount of trypsin formed was found with the sodium salts, followed in order by lithium, potassium, rubidium, and caesium.

The experimental data for the formation of trypsin from trypsinogen in the presence of salts of the alkaline earths are given in Fig. 4. The velocity constant for the formation of trypsin (Table IV) increases with the addition of calcium, strontium, barium, and magnesium. Barium increases the velocity constant for the formation of inert protein while calcium, strontium, and mag-

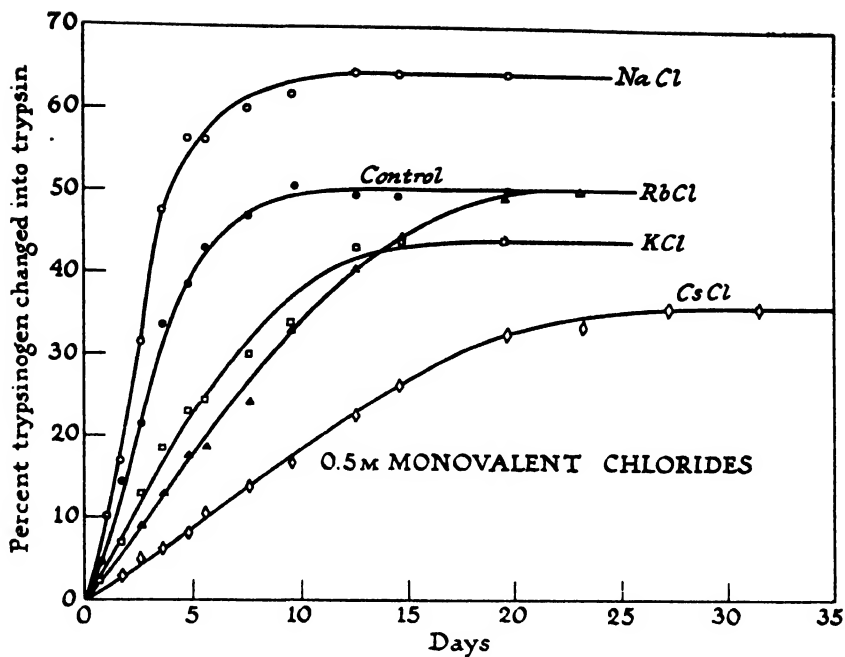


FIG. 3a

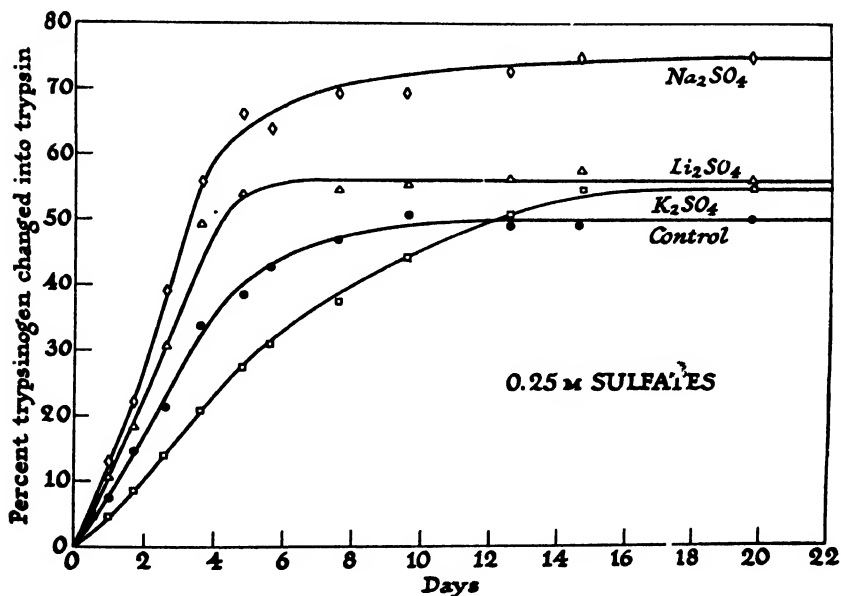
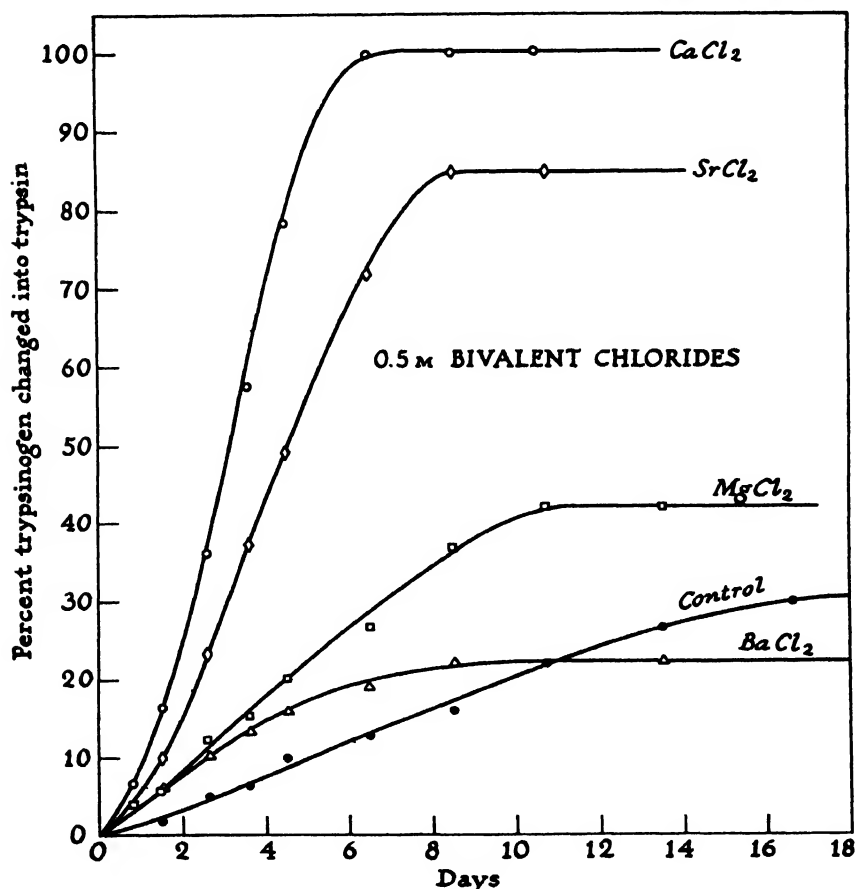


FIG. 3b

FIGS. 3a and 3b. Effect of monovalent cations on the formation of trypsin. Activation mixtures: solutions of salts of the alkali metals in 0.1 M phosphate buffer pH 7.6 and containing 0.01 mg. trypsinogen protein nitrogen per ml. Final pH = 7.1.

**TABLE III**  
*Effect of Salts of the Alkali Metals*

Salt	Molar concentration	Per cent trypsinogen changed into trypsin (observed)	$K_1$	$K_2$	$\frac{K_1}{K_2}$
<i>per mg. P.N. per day</i>					
Control.....		50	80	79	1.01
Lithium sulfate.....	0.25	57	99	75	1.32
Sodium " .....	0.25	75	115	37	3.1
Potassium " .....	0.25	55	53	45	1.18
Sodium chloride.....	0.5	64	97	55	1.76
Potassium " .....	0.5	44	52	67	0.78
Rubidium " .....	0.5	50	40	40	1.00
Caesium " .....	0.5	36	26	47	0.55



**FIG. 4.** Effect of bivalent cations on the formation of trypsin. Activation mixtures: solutions of the chlorides of the alkaline earths in 0.1 M borate buffer pH 8.0 and containing 0.01 mg. trypsinogen protein nitrogen per ml. Final pH = 7.1.

nesium decrease it. The ultimate amount of trypsin formed is therefore greater in the presence of calcium, strontium, and magnesium chloride than in the control and smaller in the presence of barium chloride. It is evident that the different cations produce as wide a variation in the results as do the different anions.

4. *Effect of Calcium Ion on the Formation of Trypsin.*—The action of calcium salts on the transformation of trypsinogen into trypsin is unique in that of all the ions studied only calcium completely inhibited the formation of inert protein. In the presence of calcium salts, even in concentrations as low as 0.02 M, trypsinogen is converted quantitatively into trypsin<sup>4</sup> and the formation follows the course of a pure autocatalytic reaction.

(a) *Effect of Varying the Concentration of Calcium Chloride.*—The effect of various concentrations of calcium chloride on the transformation of trypsinogen into trypsin and inert protein is shown in Fig. 5 and Table V. The smooth

TABLE IV  
*Effect of 0.5 M Alkaline Earth Chlorides*

Salt	Per cent trypsinogen changed into trypsin (observed)	$K_1$	$K_2$	$\frac{K_1}{K_2}$
		<i>per mg. P.N. per day</i>		
Control.....	31	26	57	0.46
Magnesium chloride.....	42	40	56	0.71
Calcium ".....	100	90	0	$\infty$
Strontium ".....	85	65	11	5.90
Barium ".....	22	42	145	0.29

curves in Fig. 5 are drawn through theoretical values of  $A$  calculated by means of the equation<sup>5</sup>

$$A = A_0 \frac{\frac{A_0}{A_0 - A_0} e^{K_1 \sigma_0 t}}{1 + \frac{A_0}{A_0 - A_0} e^{K_1 \sigma_0 t}}$$

The rate of formation of trypsin increases gradually with increasing concentration of calcium chloride while the rate of formation of inert protein decreases rapidly. The value of  $K_2$  in 0.0008 M calcium chloride is only one-fifth of the value of  $K_2$  in the control. The fraction of trypsinogen changed into trypsin increases from 25 per cent in the control to 65 per cent in the presence of 0.0008 M and to 99 per cent in 0.02 M calcium chloride.

<sup>4</sup> The trypsin formed can be easily crystallized; the procedure will be described in a future publication.

<sup>5</sup>  $e$  is the Napierian constant; the other symbols are defined in footnote 2.



(b) *Effect of Varying the Concentration of Trypsinogen.*—Fig. 6 *a* shows the curves for the formation of trypsin from various concentrations of trypsinogen in the presence of 0.1 M calcium chloride. In each case all of the trypsinogen is changed into trypsin and no inert protein is formed. Within the range of

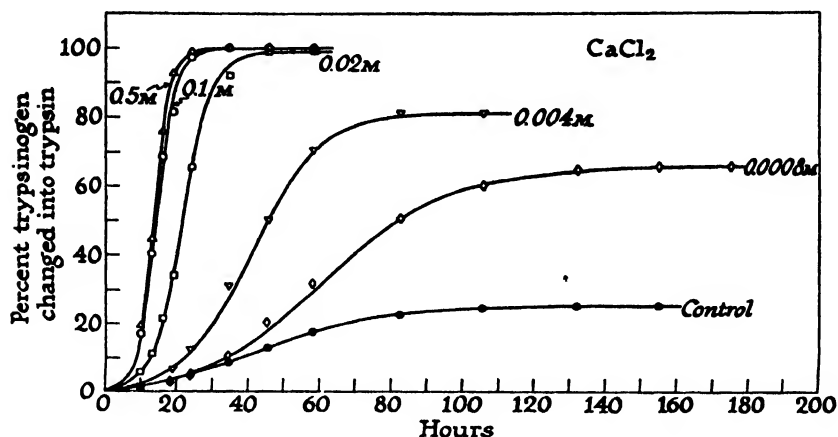


FIG. 5. Effect of concentration of calcium chloride on the formation of trypsin. Activation mixtures: various concentrations of calcium chloride in 0.1 M borate buffer pH 8.0 and containing 0.1 mg. protein nitrogen per ml. Final pH = 7.2. Smooth curves drawn through calculated points.

TABLE V  
Effect of Calcium Chloride

Molar concentration calcium chloride	Per cent trypsinogen changed into trypsin (observed)	$K_1$	$K_2$	$\frac{K_1}{K_2}$
<i>per mg. P.N. per day</i>				
0	25	12	37	0.33
0.0008	65	13	7	1.86
0.004	81	25	6	4.17
0.02	99	58	0.6	97.0
0.1	100	90	0	$\infty$
0.5	100	104	0	$\infty$

concentrations of trypsinogen used, doubling the concentration of trypsinogen doubles the rate of formation of trypsin. The values of  $\log \frac{A}{G_0 - A}$  vs.  $t$ , for the same series are plotted in Fig. 6 *b*. The experimental points lie on straight lines, the slopes of which are proportional to the initial concentrations of trypsinogen used in agreement with the theory of the kinetics of a simple unimolecular autocatalytic reaction.

The curves for the rate of formation of trypsin from various concentrations of trypsinogen in the presence of 0.02 M and 0.001 M calcium chloride are given

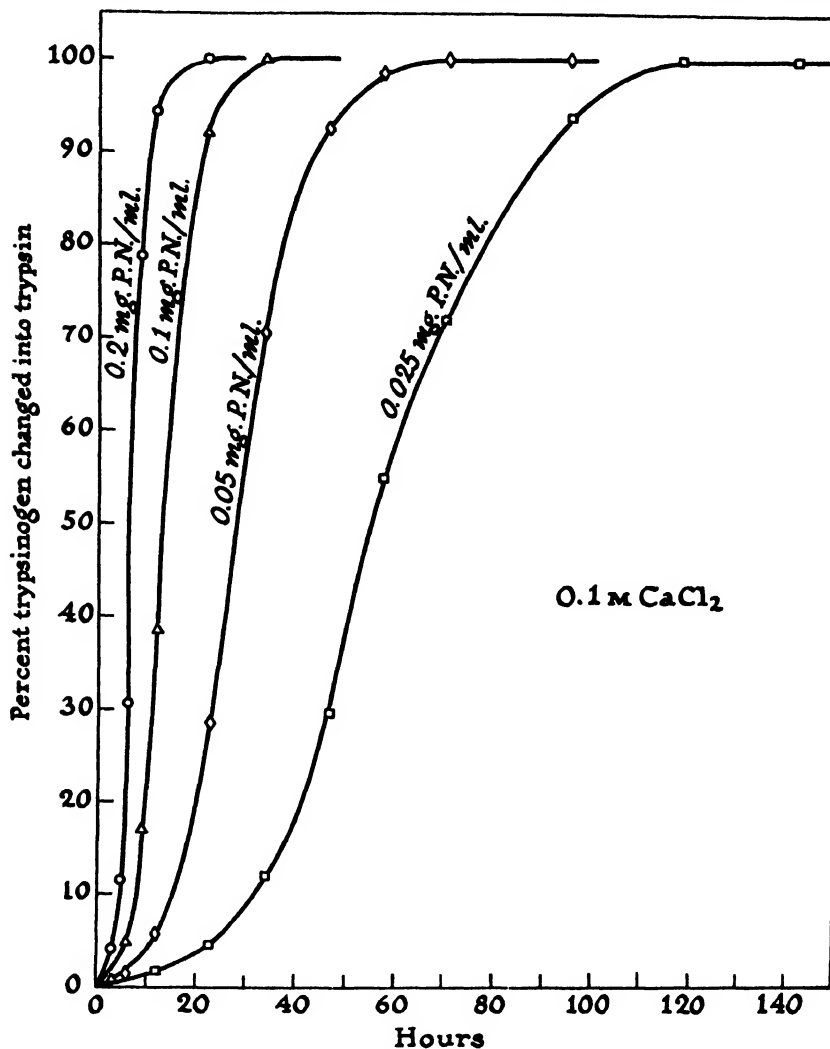


FIG. 6a

FIGS. 6 a, 6 b, 6 c, 6 d. Effect of concentration of trypsinogen on the formation of trypsin. Activation mixtures: calcium chloride solutions in 0.1 M borate buffer pH 8.0 and containing various amounts of trypsinogen per ml. Final pH = 7.2.

in FIGS. 6 c and 6 d. Although here the concentrations of the salt are insufficient to inhibit completely the formation of inert protein, the percentage of trypsinogen changed into trypsin is independent of its initial concentration in

the case of 0.02 M calcium chloride and even increases with increasing concentration of trypsinogen in 0.001 M. It appears from these results that the effect of calcium in preventing the formation of inert protein is not due to the formation of an undissociated stoichiometric compound between it and the

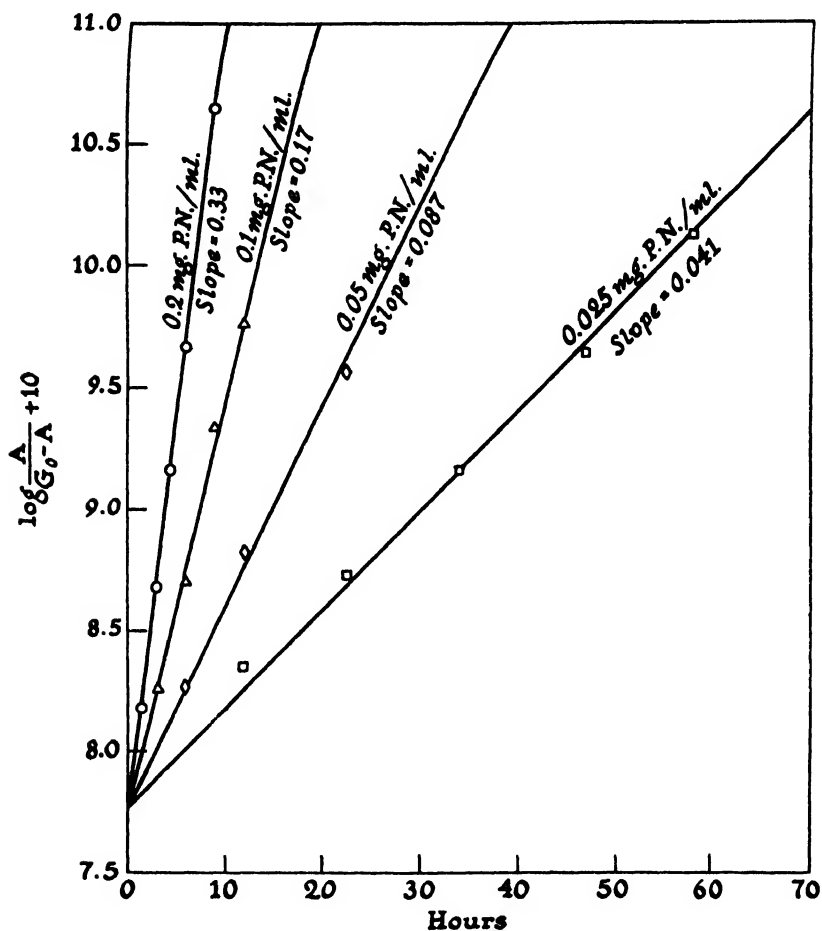


FIG. 6b

trypsinogen since in that case the percentage of trypsinogen changed into trypsin would decrease as its concentration increased.

(c) *Effect of Calcium Chloride at Various pH.*—Figs. 7a and 7b give the curves for the formation of trypsin from trypsinogen in the presence and absence of calcium chloride at various pH. In both cases the rate of formation of trypsin increases with increasing pH. In the absence of calcium chloride, however, the ultimate amount of trypsin formed decreases rapidly with increase

in pH due to the larger amounts of inert protein formed in the alkaline solutions (Kunitz, 1939), whereas in the presence of calcium chloride the transformation of trypsinogen into trypsin is complete over the whole range of pH used.

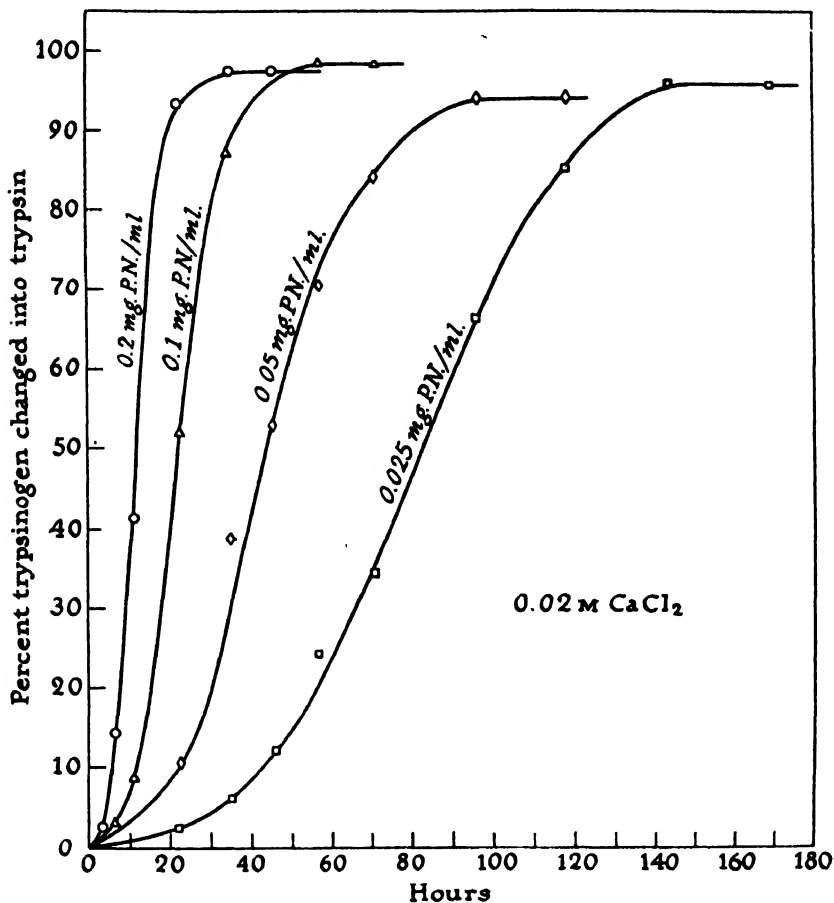


FIG. 6c

(d) *Effect of Various Calcium Salts.*—Fig. 8 gives the experimental data for the formation of trypsin in the presence of various calcium salts. The trypsinogen is converted quantitatively into trypsin in all cases and the rate of formation of trypsin is approximately the same regardless of the anion used. Evidently the action of the calcium ion predominates, masking the action of the anion.

(e) *Effect of Calcium Chloride on the Action of Trypsin.*—Calcium chloride

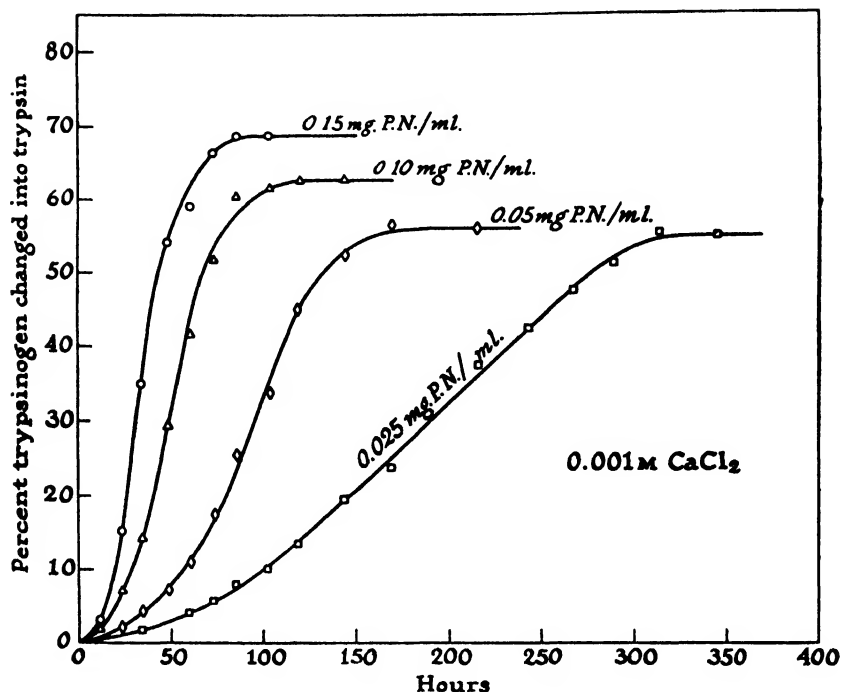


FIG. 6d

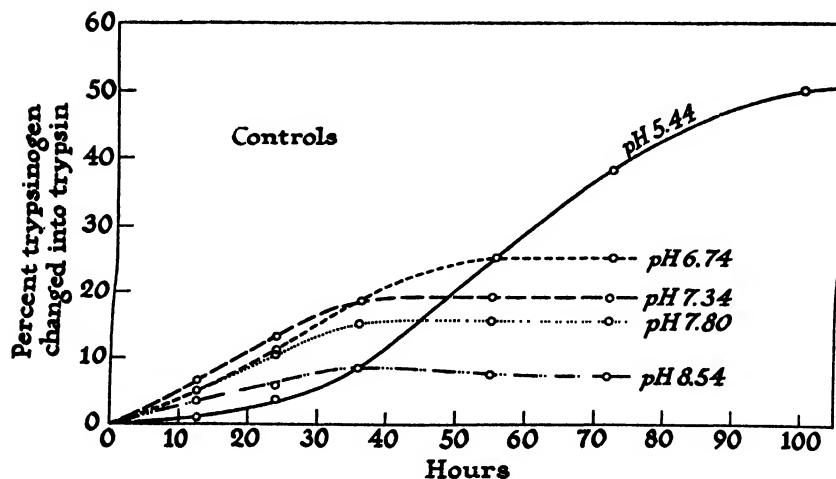


FIG. 7a

FIGS. 7a and 7b. Effect of pH on the formation of trypsin. Activation mixtures: 0.02M acetate-barbiturate buffers of various pH in 0.02M calcium chloride or water and containing 0.1 mg. trypsinogen protein nitrogen per ml.

in concentrations sufficient to inhibit completely the formation of inert protein and to accelerate the rate of formation of trypsin was found to have practically

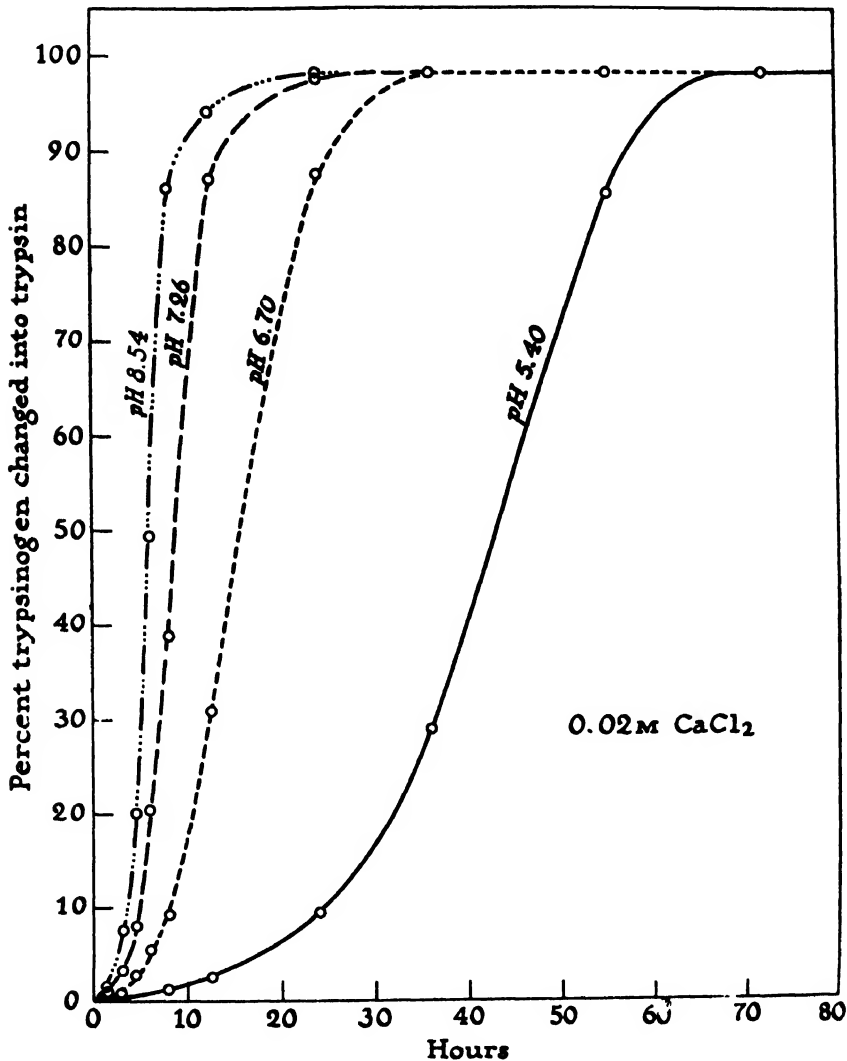


FIG. 7b

no effect on the tryptic digestion of hemoglobin or of benzoyl-L-arginine amide nor on the formation of chymotrypsin from chymotrypsinogen by means of trypsin. This together with the fact that the influence of calcium chloride on the formation of trypsin from trypsinogen is exactly the opposite of its

influence on the formation of inert protein, although both reactions are catalyzed by trypsin, leads one to assume that the action of calcium chloride is on the trypsinogen rather than on the trypsin, or else that the various actions of trypsin are quite different in character.

(f) *Effect of Calcium Chloride on Enterokinase and Mold Kinase.*—The rate of formation of trypsin from trypsinogen as catalyzed by enterokinase (Kunitz,

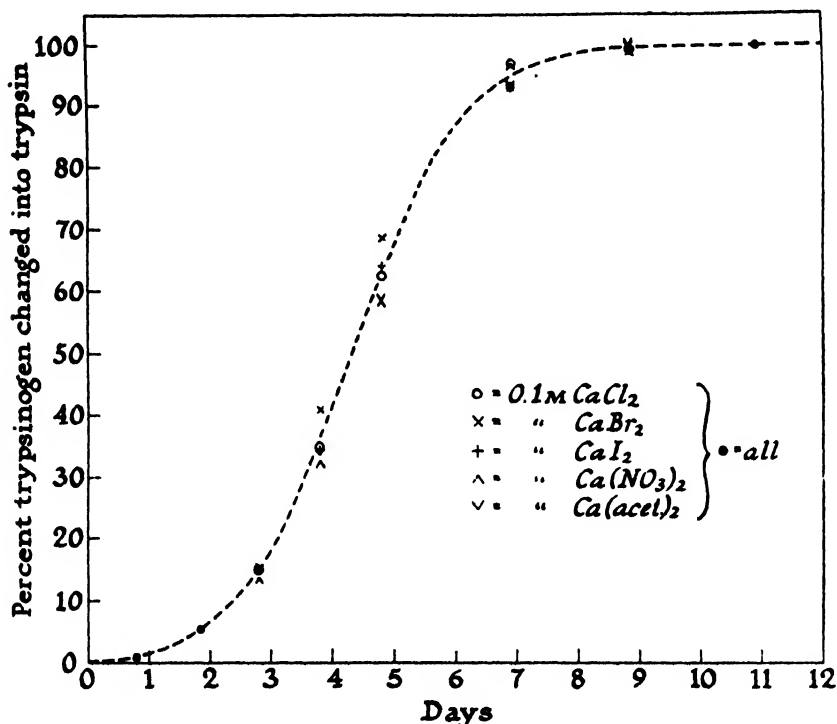


FIG. 8. Effect of various calcium salts on the formation of trypsin. Activation mixtures: 0.1 M solutions of various calcium salts in 0.1 M borate buffer pH 8.0 and containing 0.01 mg. trypsinogen protein nitrogen per ml. Final pH = 7.5.

1939 *a*) and by mold kinase (Kunitz, 1938) was found to be depressed in the presence of 0.1 M calcium chloride.

#### SUMMARY

Crystalline trypsinogen is completely transformed into trypsin by means of trypsin in the presence of calcium salts. The process follows the course of a pure autocatalytic unimolecular reaction.

In the absence of calcium salts, the autocatalytic formation of trypsin from trypsinogen is complicated by the transformation of part of the trypsinogen

into an inert protein which cannot be changed into trypsin by any known means.

Salts increase or decrease the rate of both reactions so that the ultimate amount of trypsin formed varies with the nature and concentration of the salt used. With equivalent concentrations of salt the percentage of trypsinogen changed into trypsin is greatest in the presence of calcium ion followed in order by strontium; magnesium and sodium; rubidium, ammonium, lithium, and potassium; caesium and barium. With the anions the largest percentage of trypsinogen transformed into trypsin was found with the acetate, sulfate, oxalate, citrate, tartrate, fluoride, and chloride ions followed in order by bromide, nitrate, and iodide.

The formation of inert protein is completely suppressed by concentrations of calcium ion greater than 0.02 M.

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# ELECTRIC POTENTIAL AND ACTIVITY OF CHOLINE ESTERASE IN THE ELECTRIC ORGAN OF ELECTROPHORUS ELECTRICUS (LINNAEUS)

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## INTRODUCTION

According to the theories of Loewi and of Dale acetylcholine (ACh) acts as the specific transmitter substance of nerve impulses to an effector organ or to a second neuron. Recent investigations suggest that the original theory must be altered to account for the ACh metabolism which closely parallels the electrical changes occurring everywhere at or near the neuronal surface (Nachmansohn and Meyerhof, 1941). This interpretation was necessary to explain the following observations.

1. A high concentration of choline esterase exists in strong electric organs (*Torpedo*, *Gymnotus electricus*). These organs can split in 60 minutes an amount of ACh equivalent to 1-3 times their own weight. The concentration of the enzyme is of the same order of magnitude as that estimated previously for motor end plates of muscle. The essential point is the fact that in these organs considerable amounts of ACh can be split during the refractory period which is of the order of milliseconds. This makes possible the assumption that ACh is closely connected with the discharge. The prerequisite for such an assumption is the possibility of a quick removal of the active substance. The existence of such a high concentration of the enzyme appears particularly significant in view of the high water (92 per cent) and low protein content (2-3 per cent) of the organs. In the weak electric organ of Ray the concentration is relatively low. If in the three species number of plates per centimeter and E.M.F. per centimeter are compared with the concentration of the enzyme a close parallelism is obtained (Nachmansohn, 1940). The observations on the concentration of choline esterase in electric organs, established during the years 1937 and 1938, and simultaneous and independent studies of Auger and Fessard on the discharge of electric organs under different conditions, especially after degeneration of the nerve, led to investigations on *Torpedo*

\* This work was made possible by a grant of the Dazian Foundation.

*marmorata* carried out in Arcachon. There it was demonstrated that ACh is present in high amounts in the electric organ, that after nerve stimulation ACh is liberated in these organs, and that injection of ACh into the organ produces a discharge (Feldberg, Fessard, and Nachmansohn, 1940). These experiments support the view suggested by the previous experiments that the discharge is connected with the appearance of ACh.

2. Only a quantitative difference exists between the concentration of the enzyme in nerve fibers and that at synapses. This difference was interpreted as being related to a concentration of the enzyme at or near surfaces and therefore high at synaptic regions where the end arborization increases the surface (Couteaux and Nachmansohn (1940)). Direct evidence for this view was obtained with the giant fiber of squids in which it was found that practically all the enzyme is localized in the sheath and that only negligible amounts occur in the axoplasm (Boell and Nachmansohn, 1940).

The data on the concentration of choline esterase in the electric organ of *Electrophorus electricus* (Linnaeus) (= *Gymnotus electricus*) were incomplete and unsatisfactory. Only one specimen had been examined and only two determinations were made at that time.<sup>1</sup> Among all species the electric organ of *Electrophorus* has the greatest E.M.F. A detailed study of the enzyme concentration and distribution in relation to the E.M.F. appeared necessary. The present paper fills this gap and offers new evidence for the parallelism between number of plates per centimeter, E.M.F. per centimeter, and enzyme activity.

### Methods

The activity of choline esterase was determined with the Barcroft-Warburg manometric method in the same way as described previously (Nachmansohn, 1939). Frog's Ringer solution with bicarbonate buffer was used as medium. The tissue was very thoroughly ground; for it is important to have a homogeneous suspension because of the high esterase activity of electric organs. Although the pieces of tissue taken for grinding were small, the suspension had to be diluted to a large volume, and the 3 cc. put into the Warburg vessel were only a small fraction of the total suspension. For instance 3 cc. of the suspension prepared from pieces of the electric organ of the smaller eel contained only about 0.5 mg. fresh tissue. The weights given in the tables indicate the fresh weight of tissue ground. As the amount actually put into the vessels was approximately the same in each series of experiments, that for each determination is not specified separately but is indicated only for each table. The balance used permits rapid and precise weighing which is necessary for such small amounts of fresh tissue. The balance has magnetic damping on both sides so that in about 15 seconds 0.1 mg. can be read on a scale directly and 0.01 mg. can be estimated. Generally only 4 manometric readings were made in intervals of 5 minutes

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<sup>1</sup> This specimen died in October, 1938, in the Institut Océanographique in Paris and the enzyme activity was determined by one of us (D. N.) in the Laboratoire de Physiologie Générale de la Sorbonne.

after the substrate had been added to the enzyme suspension. The first 5 minute reading was not included in the calculation.

The protein determinations of the enzyme solutions were carried out gravimetrically. To 1 cc. of the enzyme solution in a 3 cc. centrifuge tube there was added 1 cc. of 40 per cent trichloroacetic acid. After 24 hours the precipitate was centrifuged for 30 minutes in a centrifuge at a rate of 4000 R.P.M. The precipitate was washed three times with distilled water. The centrifuged tube was put into a drying oven at 105°C. for several hours and the weight was then determined.

## RESULTS

### *I. Electric Organ*

The electric organs of two eels were examined. The first was medium sized and had a length of 120 cm., the second was small and only 67 cm. long.

The electric organ of the larger specimen had a length of 78 cm. Pieces were taken successively at five points from the head to the caudal end of the organ and the concentration of choline esterase was determined. The values obtained are given in Table I. The highest concentration of the enzyme is found in the region near the head end of the organ; it decreases continuously towards the caudal end. Fig. 1 shows the tentative curve of the change in enzyme concentration. The shape of this curve appears to be analogous to that which indicates the E.M.F. per centimeter and number of plates per centimeter determined on an eel of approximately the same size (Cox, Rosenblith, Cutler, Mathews, and Coates, 1940).

The number of sections (five) at which the enzyme concentration was determined and the number of determinations in each section were not sufficient for ascertaining the precise shape of the curve. In each section the concentration varies from one piece to the other: In the section 45 cm. from the snout four determinations were made, and the QCh. E. values were found to vary from 83-112. Such differences are not surprising. As the experiments quoted in the introduction suggest, choline esterase is concentrated at surfaces of neurons. The amount of active surface will vary from one piece to another. The greatest part will be presumably at the innervated side of the disc. It can be expected, therefore, that even in pieces taken from the same region the enzyme concentration will differ according to the amount of active surface although the number of plates per centimeter is equal. If a sufficient number of large pieces were taken these differences might become insignificant, and the value obtained would approach the average value of that section. But it is difficult to grind large pieces homogeneously and the error due to this difficulty may be as great as that due to the variations of active surface in small pieces.

In order to get more information about the variations which may occur in a given piece due to the uneven distribution of active surface, the enzyme concentration was determined in a series of slices cut with a freezing microtome. The results are given in Table II. Pieces I and II were taken about 60 cm.

TABLE I

Concentration of choline esterase in sections of the electric organ of *Electrophorus*, taken successively from the head to the caudal end. Length of animal 120 cm. *D* = distance of section from the snout in cm. *W* = mg. tissue ground. 2-5 mg. fresh tissue were taken per vessel. QCh.E. *s* = single determination, *a* = average.

<i>D</i>	Main organ			Organ of Hunter		
	<i>W</i>	QCh.E.		<i>W</i>	QCh.E.	
		<i>s</i>	<i>a</i>		<i>s</i>	<i>a</i>
30	66.0	134.0	125.0	73.0	93.5	93.5
		118.0			93.5	
		123.0				
	68.5	135.0 134.0	134.5	65.0	107.0 103.0	106.0
45	46.0	110.0	99.0	21.2	107.0	107.0
		93.0				
		103.0				
	48.7	110.0	112.0	28.0	140.0	132.5
		111.0			125.0	
		116.0				
	66.0	92.0	83.0			
		76.0 82.0				
	60.0	100.0 93.0	96.5			
60	47.0	50.0	80.0	44.5	79.0	76.5
		50.0			73.7	
	65.5	58.2	62.0	44.0	64.5	65.8
		65.7			67.0	
68	140.0	35.0	33.0	98.7	29.0	39.0
		31.0			29.0	
	165.0	38.0	40.0	76.0	27.0	27.5
		42.0			28.0	
102				41.0	37.5 41.5	39.5
108	71.0	36.5 42.5	39.5	45.0	42.5 42.0	42.5

from the snout of the eel described above. Piece III was cut out by biopsy from an eel of similar size and the region chosen was near to the head end of the organ. The values show how great the variations are from one slice to the other. Of special interest are the values obtained with piece I. It seems as if the values vary in a certain rhythm. This is demonstrated in Fig. 2. It may be assumed that this rhythmic change is not incidental but corresponds to a rhythmical change in active surface. This may be easily understood from the histological structure. It may appear only at a certain thickness of slices and when the slices are cut in a direction exactly parallel to the discs,

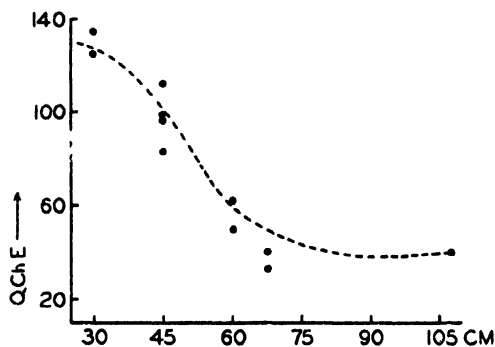


FIG. 1

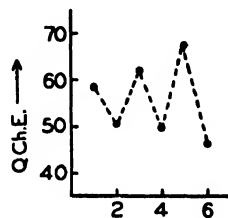


FIG. 2

FIG. 1. Concentration of choline esterase in different sections of the electric organs of the electric eel, *Electrophorus electricus* (Linnaeus), taken successively from the head to the caudal end. Length of the animal 120 cm. Abscissae: QCh.E. values. Ordinates: Distance of the section from the snout in centimeters.

FIG. 2. Concentration of choline esterase in successive slices of a piece of electric tissue cut with a freezing microtome. Abscissae: QCh.E. values. Ordinates: Number of the successive slices.

There are three superimposed organs on each side: the main organ, Hunter's organ, and the bundle of Sachs which forms the greater part of the posterior section. In the bundle of Sachs number of plates per centimeter and E.M.F. per centimeter increase towards the caudal end (Cox, Rosenblith, Cutler, Mathews, and Coates, 1940), making the conditions more complex. It may be that the pieces at a distance of 68 cm. from the snout belonged to the bundle of Sachs and that therefore the values were low, even lower than at the caudal end. When these determinations were made, no attention was paid to this point. In the organ of Hunter the concentration of the enzyme does not differ appreciably from that in the main organ. This again is in agreement with the number of plates per centimeter.

In spite of all restrictions pointed out the shape of the three curves: (I)

TABLE II

Concentration of choline esterase in successive slices cut with a freezing microtome. Piece I and II were taken from a section 60 cm. from the snout; same eel as in Table I. Piece III was taken from an eel of similar size; the section was near the head end of the organ.

	I		II		III	
Thickness of slices in $\mu$ .....	150-200		100		150-200	
	Slice No.	W	QCh.E.	W	QCh.E.	W
1	6.3	57.0 60.0 50.0	2.3	70.5	2.8	125.0
2	11.8	51.0 62.0	5.0	56.3	2.4	88.0
3	8.8	62.0	6.6	70.5	—	—
4	12.1	49.5	2.7	82.0	3.1	132.0
5	9.4	67.5	2.2	76.0	3.9	125.0
6	9.8	46.2			4.5	103.0
7					5.3	90.0
8					5.5	90.0
9					5.7	87.0

TABLE III

Concentration of Choline Esterase in the Electric Organ of an *Electrophorus* of 68 Cm. Length

D	W	QCh.E.		D	W	QCh.E.		D	W	QCh.E.	
		s	a			s	a			s	a
17	17.5	612.0 648.0	630.0	25	12.4	638.0 677.0	658.0	39	15.3	530.0	530.0
	10.2	682.0 697.0	690.0		7.3	567.0	567.0		10.9	394.0	394.0
					14.5	520.0 500.0	510.0		12.8	460.0	460.0
	6.9	615.0	615.0					53	16.9	191.0 187.0	189.0
20	14.0	620.0	620.0	32	15.2	610.0	610.0		12.1	115.0 114.0	115.0
	11.8	720.0 680.0	700.0		16.3	630.0 633.0	632.0		8.0	161.0 139.0	150.0
	11.7	740.0 750.0	745.0		12.9	680.0 685.0	683.0		20.4	150.0 153.0	152.0
					15.2	690.0	690.0		11.4	149.0	149.0
	10.1	745.0 690.0	718.0					59			
	11.1	735.0 740.0	738.0								

D = Distance from snout in cm.

W = mg. tissue ground.

QCh.E. s = single determination, a = average.

The amount of tissue taken per vessel was about 0.5 mg.

enzyme concentration, (II) number of electric discs per centimeter, and (III) E.M.F. per centimeter seems to be essentially the same. This becomes even more obvious by the experiment carried out on the smaller specimen. The length of the electric organ in this animal was 52 cm. The enzyme concentration was determined at seven sections and more samples were taken from each section than in the first animal examined. The results are given in Table III. The S-like form of the curve is here quite obvious (Fig. 3).

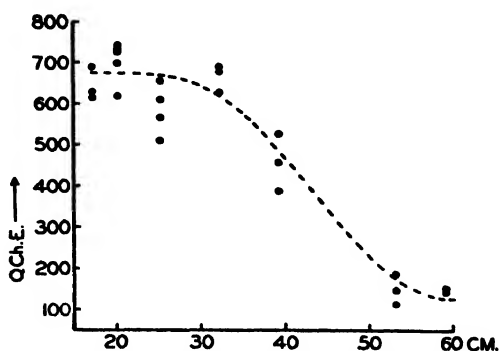


FIG. 3. The same as in Fig. 1, carried out on the specimen of 68 cm. length.

TABLE IV

Concentration of choline esterase in different sections of the electric organ of the *Electrophorus* of 68 cm. length at the 8th day following death. The symbols used are the same as those in Tables I and III. b.o.S. = bundle of Sachs.

D	W	QCh.E.	D	W	QCh.E.
22	17.5	608.0	50	11.3	410.0
			(Main org.)		
29	12.8	818.0	50	8.7	220.0
			(b.o.S.)		
36	15.6	690.0	55	11.0	250.0
43	17.9	550.0			

The E.M.F. per centimeter *versus* distance from the snout has not been determined accurately as in the larger animal. But the observations available indicate that the shape of the curve is the same (Coates, Cox, and Granath, 1937). The values of QCh.E. are around 6–700 near the head end and around 150 near the caudal end as compared with 120–140 and around 40 respectively. The determinations were made a few days following death. Although the animals were kept in the ice box at 1–2°C., it may be that the absolute values were a little lower than those found. The enzyme remains stable for many months (Nachmansohn and Lederer, 1939). But the tissue in view of its peculiar high water content may lose some water, and the real weights may be a little higher than those actually found. In Table IV the QCh.E. values are



given of samples taken at different sections 2 days later than those of Table III. The values are definitely a little higher. When these samples were cut out it appeared that the tissue did not retain its water as well as in the first few days following death. But if the absolute values may be a little too high even for the first days, they are comparable in any case for the smaller and larger specimen because the determinations were made during the same period following death.

In the section at a distance of 50 cm. from the snout the bundle of Sachs could be clearly distinguished from the main organ, and samples were taken separately in this case. The concentration in the main organ is nearly twice as high as in the bundle of Sachs: 410 and 220 respectively (Table IV).

## II. Central Nervous System

It was always believed that the electricity of electric organs is not extraordinary as compared with that of ordinary nerves, and that it is only the arrangement of the plates in series by which these organs are distinguished and by which the great E.M.F. is attained (Du Bois-Reymond, 1877; Burdon Sanderson and Gotch 1889). It is therefore interesting to know whether the concentration of choline esterase in the nerves supplying the electric organ differs from that in ordinary nerves. In *Torpedo marmorata* the enzyme concentration of electric lobes and the nerves innervating the electric organ appears to be higher than in the rest of the brain and in motor nerves (Feldberg, Fessard, and Nachmansohn). But in determinations carried out on *Torpedo occidentalis* Storer no significant difference was found between the QCh.E. values in the electric lobe and its nerves and other parts of the central nervous system (Nachmansohn and Meyerhof, 1941). These experiments did not support the assumption that the nerves supplying the electric organ are distinguished from ordinary nerve by a special concentration of choline esterase.

In order to get more information about this question the enzyme concentration was determined in brain and spinal cord of *Electrophorus* and compared with that in a gold fish (*Carassius auratus*). The *Electrophorus* belongs to the series *Ostariophysi* like the *Cyprinidae* among which are classified the carps and the gold fishes. The specimen examined was the larger eel. Whereas in *Torpedo* there are special centers, the electric lobes, which innervate the electric organs, the *Electrophorus* has no such centers. The cell bodies of the nerves innervating the electric organs are located in the spinal cord around the central canal. These cells begin to appear in the spinal cord not far from the medulla oblongata; their number increases toward the middle of the spinal cord and then decreases again. The spinal cord was therefore examined at several distances from the medulla.

The values obtained are given in Table V. The QCh.E. values of the spinal cord of *Electrophorus* are rather low. They do not differ at various distances

from the medulla. In the spinal cord of the gold fish they are four times as high. In the central nervous system the values of the two species are similar. Only in the hypothalamus of the gold fish are they particularly high: 40.0 and 44.0 as compared with 16.8 in the *Electrophorus*.

### III. Striated Muscle

Electric organs are considered as modified muscle end plates phylogenetically evolved by transformation of striated muscle. Since the time that Babuchin

TABLE V  
Concentration of Choline Esterase in Brain and Spinal Cord of *Electrophorus* and *Carassius auratus*

Part of C.N.S.	W	QCh.E.	Part of C.N.S.	W	QCh.E.
<i>Electrophorus</i>					
Left cerebral hemisphere	50.5	7.2	Hypophysis	25	4.6
Right cerebral hemisphere	55.5	8.9	Medulla obl.	39.5	9.4
2 optic lobes	75.0	12.4	Spinal cord close to	24.0	5.3
Cerebellum	124.0	12.5	Spinal cord 30 cm. from	48.0	2.8
Area acustico—later	221.4	9.8	snout		
Hypothalamus	45.0	16.8	Spinal cord 60 cm. from	53.0	3.0
			snout		
			Spinal cord 75 cm. from	24.0	3.0
			snout		
<i>Carassius auratus</i>					
1 cerebral hemisphere	25.9	6.0	Hypothalamus	52.6	40.0
2 optic lobes	80.3	15.2			44.0
Cerebellum	67.6	10.7	Base of mid-brain	27.0	11.0
2 vagal lobes	90.8	15.8	Medulla obl.	83.8	16.9
		16.7	Spinal cord	42.0	12.5
					12.2

studied the ontogenetical development of *Torpedo* and described how the electric plates gradually develop from embryonic muscle fibers, there has been no doubt as to the genetic relations between the two tissues. Every possible transition has been observed between the phylogenetically lowest to the highest form, especially in weak electric organs which are not so highly differentiated as the strong organs, and in which the discs may retain their muscular characteristics even when the formation is perfect.

Quite different groups of striated muscles can be transformed into electric organs. In *Gymnotus* the deepest part of the ventral trunk muscles is transformed into the large electric organs except the "intermediate muscular layer" (Biedermann). It was thought possible that striated muscles near the electric

organ might be in a state intermediate between striated muscle and electric organ, and that in this case the concentration of choline esterase in these muscles might be higher. Therefore the enzyme activity was determined in a few samples of the dorsal trunk muscles and in the intermediate layer. The determinations were carried out on the smaller eel. The data obtained are given in Table VI. Only a few experiments were carried out but they clearly indicate that the QCh.E. values are not comparable with those of ordinary striated muscle but are much nearer to those of electric organs. It appears moreover that the enzyme concentration is higher the nearer the muscles are located to the electric organs: the relatively highest QCh.E. values were found in the "intermediate layer:" 210.0 and 141.0. In the dorsal muscles the

TABLE VI

Concentration of choline esterase in muscles of *Electrophorus*. D = distance from the snout. L = location. Inf. = inferior dorsal trunk muscles, l = left, r = right side; sup. = superior dorsal trunk muscles. I.m.l. = intermediate muscle layer.

D	L	W	QCh.E.	D	L	W	QCh.E.
32	Inf. l	29.8	90.5 90.0	39	Inf. r	29.0	103.0
	Inf. r	25.8	190.0		Sup. l	18.3	29.0
	Sup. l	19.9	55.0 57.0	46	Inf. r	28.8	39.0
	I.m.l.	12.8	210.0		I.m.l.	9.4	141.0

superior group has a lower concentration than the inferior group. As far as we know no histological studies have been made on these muscles. It would be interesting to know whether these muscles are in a process of transformation towards electric organs and, if so, how far this process is developed.

#### IV. Preparation of Enzyme Solutions from the Electric Organ

Electric organs are a suitable material for the preparation of active enzyme solutions, owing to the great amount of enzyme present and the low protein content. Very active solutions were obtained from the electric organ of *Torpedo marmorata* (Nachmansohn and Lederer, 1939) and recently from *Torpedo occidentalis* Storer.<sup>3</sup> It appeared interesting to compare the solutions which can be obtained from the electric organ of *Gymnotus* with those of *Torpedo*. The preparation was carried out in the same way as that with the elec-

<sup>3</sup> Nachmansohn, unpublished experiments.

tric organ of *Torpedo*: about 500 gm. of organ from the large specimen were minced with an automatic machine. The minced tissue was ground with silicate and centrifuged about 30 minutes. About 500 cc. clear supernatant fluid were obtained. 1 cc. of this preparation split 650 mg. ACh in 60 minutes. The protein content was 11.2 mg. per cc. 1 mg. protein split therefore 53 mg. ACh in 60 minutes. In the preparations obtained in the same way from electric organs of *Torpedo* 1 cc. of the solution split 400–1500 ACh in 60 minutes 1 mg. protein split 100–200 mg. ACh per hour. After the ground tissue had been centrifuged and the supernatant fluid removed 250 cc. of 10 per cent ammonium sulfate solution were added to the extracted tissue. The tissue was ground again and centrifuged. The solution obtained in this way is more active per protein unit: 1 cc. split 487 mg. ACh per hour and contained 3.71 mg. protein. 1 mg. protein split therefore 131 mg. ACh per hour. But solutions prepared in this way from electric organs of *Torpedo* were also more active per protein unit: 1 mg. protein split 2–300 mg. ACh in 60 minutes.

The electric organs of *Torpedo* appear therefore to be a more suitable material for the preparation of enzyme solutions than those of *Gymnotus*. This was to be expected from their higher enzyme concentration. It is possible and even probable that in the organ of the small specimen where the enzyme concentration is not only higher than in the large specimen but also higher than in *Torpedo*, the solution obtained would be more active than that from the organs of *Torpedo*. But the amount of material available is rather small.

#### DISCUSSION

These experiments furnish new evidence for the parallelism between number of plates per centimeter E.M.F. per centimeter, and concentration of choline esterase. Whereas until now this parallelism was apparent only when different species were compared, here it is shown that in the same organ great variations of enzyme concentration occur which are essentially the same as those of E.M.F. per centimeter and number of plates per centimeter.

The experiments support the view that a relation exists between ACh metabolism and the intensity of the discharge. If ACh is connected with the discharge it must appear and disappear in milliseconds. If speculation were to be excluded, the only known way for removing the active substance so rapidly is by the activity of the specific enzyme choline esterase. The greater the potential difference becomes, the greater must be the amount of active substance liberated, and the higher the concentration of the enzyme. Electric organs are highly specialized in their function. The discharge is here the final event. The fact that a specific enzyme is so highly concentrated in this organ—so poor in protein—is in itself support for the assumption that the substrate is connected with its function. If we also recall (1) that ACh can produce potential differences if injected into the organ, (2) that the potential difference is much

greater if the choline esterase is inactivated by eserization of the organ, and (3) that the appearance of ACh during the discharge can be demonstrated in the electric organ (Feldberg, Fessard and Nachmansohn, 1940), the theory of a close correlation between electrical changes and ACh metabolism appears justified. As potential differences occur at surfaces (Hodgkin and Huxley, 1939) the evidence for a concentration of choline esterase at surfaces (Boell and Nachmansohn, 1940), is in this connection particularly pertinent.

The voltage developed in a discharge between two points along the electric organ depends upon the E.M.F., the current, and the internal resistance according to the equation:

$$V = E - IR$$

where  $E$  is the E.M.F.,  $I$  the current, and  $R$  the internal resistance. The special conditions of these relations in the electric organ have been recently discussed (Cox and Coates, 1938). Two assumptions can be made about the way in which ACh may act: (1) it can produce the E.M.F. directly by action on the surface, (2) it can decrease the resistance and this again by action on the surface, e.g. by increasing the permeability of the disc boundary. In either case the parallelism described here could be explained.

For the difference in enzyme concentration found between the large and small eel the following explanations appear possible. The maximal discharge varies considerably from one specimen to another even in animals of the same size. The actual discharge of the two specimens has not been recorded. It may be that the E.M.F. per centimeter was higher in the small specimen although it is not at all likely that it was four times as high. On the other hand the possibility is envisaged that ACh may act upon the resistance, and it may be that the drop in resistance per square centimeter of the disc boundaries varies with the size of the individual.

#### SUMMARY

1. If the concentration of choline esterase is determined at different sections from the head to the caudal end of the electric organ of *Electrophorus electricus* (Linnaeus) S-like curves are obtained. These curves are essentially the same as those which show the number of electric discs per centimeter and the E.M.F. per centimeter.

2. In the organ of Hunter the concentration of the enzyme does not differ from that in the adjacent parts in the main organ. This again coincides with the observations on the number of plates per centimeter in this organ.

3. The concentration of the enzyme was determined in different parts of the brain and the spinal cord and compared with that in a gold fish. The concentrations here are of the same order, but in the spinal cord of the eel the

concentration is even lower than in the gold fish. As the cell bodies of the nerves innervating the electric organ in the spinal cord, these results do not lend support to the assumption of a special concentration of the enzyme in these nerves.

4. In the muscles adjacent to the electric organ an enzyme concentration has been found which is of the order of that in the electric tissue itself and much higher than in ordinary striated muscles.

5. The suitability of the organ for the preparation of enzyme solutions has been investigated and compared with that of the organ of *Torpedo*.

It is a pleasure to express our thanks to Dr. R. G. Meader (Section of Neuroanatomy, Yale University School of Medicine) for assistance in the dissection of the central nervous system.

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# THEORY AND MEASUREMENT OF VISUAL MECHANISMS

## VI. WAVE-LENGTH AND FLASH DURATION IN FLICKER

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### I

For a square image subtending *ca.*  $6.13^\circ$  of visual angle on a side, centrally fixated, we have described the relations of flash frequency  $F$  to mean flash intensity  $I_m$  for recognition of flicker, as obtained with "white" light seen unocularly and binocularly and with various proportions of light time in the flash cycle (Crozier and Wolf, 1940-41 *a, b*). The data have been analyzed in terms of the conception that, for each of the two populations of elements of neural effects combined in the typical duplex flicker recognition contour, the fundamental form is that of a normal probability summation in  $\log I_m$ . When the "rod" and "cone" contributions show extensive overlapping, as with man, the low intensity segment of the curve is determined by the summation of a partially inhibited "rod" contribution of elements with those labelled as of "cone" origin.

The properties of these elements, defined by the summation of  $dF/d \log I$  as a function of  $\log I$ , have been studied for monocular and binocular stimulation and as a function of the light time fraction. Their quantitative relations to the wave-length composition of the light should be important in a variety of ways. Certain of these questions are considered in the present paper. It is to be expected that modification of the wave-length composition of the light will produce changes in the form of the low intensity and of the high intensity segments of the  $F - \log I$  contour, and in the relations between them. This supplies an additional means of investigating the nature of the composite curve, and of the relations of excitable neural units to the elements of discriminatory effect which they produce. At the same time it is inevitable that the data, properly interpreted, should have significance for the problem of excitation as a function of wave-length. It will be pointed out that some of the conditions required for the discussion of this matter can now be seen more concretely, and that a preliminary application of their meaning can be indicated.

For four regions of the spectrum the flicker response contour was determined with  $t_L = 0.10$  and  $t_L = 0.90$  of the cycle time, with the centrally fixated field



already used for white light. The points to which attention will be directed include the  $F_{\max}$ . and other parameters ( $\tau'$ ,  $\sigma' \log I$ ) of the  $F - \log I_m$  curve as a function of wave-length composition, the relations of these parameters to the  $t_L$  fraction, the variability of  $I_1$ , and the scatter of the indices of variation. The most recent and most extensive survey of the dependence of flicker upon spectral composition (Hecht and Schlaer, 1935-36) gives no information on most of these matters. Some important conclusions to be drawn are further supported by tests made in another part of the retina (Crozier and Wolf, 1941-42). The findings will be considered as they bear upon the nature of criteria for the allocation of "cone" vs. "rod" properties to visual data, the

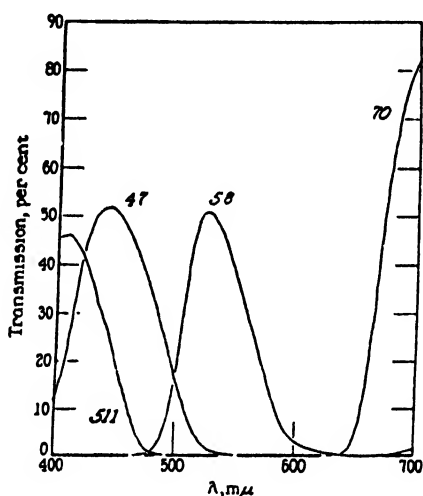


FIG. 1. Transmissions of filters used for violet, blue, green, and red

more general question of the meaning of such data for the deduction of mechanisms of the primary peripheral excitation, and for certain questions of color vision theory.

The apparatus used and the procedure followed in these observations are already described, and need not be restated here. One observer, W. J. C., was used throughout. Observations were made monocularly (left eye). For the modification of wave-length distributions use was made of Wratten filters Nos. 70, 58, 47, and Corning No. 511, giving in this order red, green, blue, and violet. The transmissions of these filters are shown in Fig. 1, as measured in a photoelectric spectrophotometer. In the tables the units of  $I$  purposely used are flash brightness units, millilamberts, determined by photometric equations with white, made below the color threshold for each light. Under our conditions these units are proportional to photons, as the ocular eye-ring provided an effectively constant "pupil" 1.8 mm. in diameter. No correction has been made for ocular transmission. For certain purposes direct energy com-

parisons with the white light are important. Such comparisons were made with a Mohl large surface thermopile and sensitive galvanometer, the thermopile being in the eye position at the discriminometer head (*cf.* Crozier and Holway, 1938-39*a*, 1939-40); these are referred to subsequently. No attempt was made to work for more monochromatic beams, since it was desired merely to modify wave-length compositions in a convenient way and at the same time to cover as large as possible an intensity range.

## II

The observations for the violet, blue, green, and red regions, at  $t_L = 0.10$  and 0.90, are given in Tables I-IV. Each  $I_m$  entry is the mean of 10, and the dispersion of these 10 is given as P.E.<sub>11</sub>. We consider first the analysis of the forms of the pair of curves for each color region, then the comparison of their properties.

For each color, as for white, the shortening of the light time fraction moves the curve toward lower intensities and increases the asymptotic maximum to which the curve rises. The curves are plotted in Figs. 2 to 5. The uppermost parts of these curves, the so called "cone" sections, are in every case well described by a normal probability summation in  $\log I_m$  (Fig. 6). As with white light, at any given wave-length composition the slopes of the two lines for  $t_L = 0.10$  and 0.90 on a probability grid are not detectably different, and thus  $\sigma_{\log I}$  is the same (although differing according to the wave-length).

In Figs. 2 to 5 the probability integrals of Fig. 6 have been continued toward  $F = 0$  and the "rod" curves obtained by difference. The observations below the kink in the  $F - \log I$  contour were taken, like those above it, in overlapping groups. Only for the *violet* did such successive series disagree. One *violet* set ( $V'$ ) with  $t_L = 0.90$  below  $F = 18$  departed widely from the others. It is given in Table I, and is analyzed graphically in Fig. 7. We tried in vain to repeat this series; the explanation for its occurrence is unknown; it cannot be accounted for by technical error. As an illustration of the kind of reproducibility otherwise experienced we may refer to an independent series made much later with the blue filter and  $t_L = 0.10$ , not given in Table II but already given in the preceding paper (Crozier and Wolf, 1940-41 *b*).

The rising and the falling parts of the "rod" contribution dissected out in this way (Figs. 2 to 5) are transferred to a probability grid in Fig. 8. Again as with white light, the two rising branches have apparently the same  $\sigma'_{\log I}$  for each color, so far as can be told, and the falling branches do not. The "rod"  $F_{\max}$ . falls (*violet, blue, green*) or rises (*red*) as  $t_L$  is increased; this results from the difference in shape and in degree separation of the several "cone" and fundamental "rod" curves (*cf.* Crozier and Wolf, 1940-41 *b*).

As with white light,  $F_{\max}$ . ("cone") declines with increase of the light time fraction, and  $\tau'$  decreases. These relations are shown in Figs. 9 and 10. The rates of change (here assumed to be, as already shown in greater detail for *white*, and as checked by incomplete data on *red* and *blue* at  $t_L = 0.50$ , recti-

TABLE I

*Violet*log  $I_m$  (ml.) and log P.E.<sub>1</sub>; an exceptional set given separately

F per sec.	$t_L = 0.10$		$t_L = 0.90$		$t_L = 0.90$	
	log $I_m$	log P.E. <sub>1</sub>	log $I_m$	log P.E. <sub>1</sub>	log $I_m$	log P.E. <sub>1</sub>
2			5.5573	7.9749	4.0247	6.6778
3			5.6641	6.2054		
			5.6614	6.1654		
4			5.8419	6.4432	4.8366	6.8300
5			5.9881	7.5241		
			5.9667	6.4193		
6			4.1044	6.5746	4.6379	5.1942
7			4.3079	6.6429		
			4.3098	6.9135		
8	5.8362	7.7955	4.5128	6.8271	3.0086	5.4816
9			4.6542	6.9428		
10	5.7419	6.2520	4.8916	5.4069	3.3498	5.6243
11			4.9973	5.2475		
12	4.004	6.4326	3.1463	5.6062	3.6020	5.9052
13			3.2335	5.5771		
14	4.2285	6.7256	3.3573	5.8726	3.8815	4.4519
15			3.4966	5.9058		
			3.4760	4.0250		
			3.5051	4.7116		
16	4.5614	5.2142	3.6711	4.1621	2.1540	4.6558
17			3.7723	4.3524		
18	4.7993	5.2192	3.8998	4.4272	2.4100	4.8476
19	4.9426	5.4081	2.0241	4.4848		
20	3.5833	4.1159	2.1856	4.9294	2.6204	3.1807
	3.5698	4.1828	2.1884	4.4827	2.6273	3.0764
	3.6039	5.6769				
	3.1125	5.6771				
21	3.3330	5.7619	2.3771	4.9902		
22	3.6653	4.6653	2.7091	3.1508		
			2.7490	3.3212		
25	3.8658	4.3531	1.0289	3.5099		
	3.8726	3.1469	1.0049	3.5270		
	3.8682	4.3071	2.9911	3.5183		
27			1.1735	3.8184		
30	2.2086	4.7355	1.3593	3.8828		
	2.1958	3.2666	1.3167	3.7121		
35	2.5912	3.0676	1.6213	3.9221		
	2.6226	2.0270	1.6081	3.9626		
40	1.0429	3.5765	0.0239	2.4921		
	1.0671	2.2560	0.0280	2.5647		
	2.9960	3.5647				

TABLE I—*Concluded*

F per sec.	$t_L = 0.10$		$t_L = 0.90$		$t_L = 0.90$	
	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$
45	1.3638	3.8778	0.4855	2.8859		
	1.4140	3.9708				
	1.3583	2.1807				
	1.3483	2.5168				
	1.3573	3.7753				
50	1.7513	2.1001	1.0465	1.8354		
	1.9733	2.3908				
	1.9186	2.4444				
	1.7640	2.5544				
	1.7720	2.9639				
52	1.8184	2.2654	1.9399	0.2420		
	0.0375	2.6018				
55	0.5217	1.4737				
	0.4595	2.9663				
	0.4408	2.9642				
58	0.3113	2.6541				
	0.3476	2.8726				
	0.3455	2.8851				
	0.4521	1.0080				
	0.3960	2.6186				
59	1.0165	1.9665	3.0742	1.7325		
	0.9806	1.4362				
	0.9859	1.3921				
	0.9600	1.5183				
	0.9370	1.1520				
60	1.2715	1.7580				
	2.9730	1.8201				
	3.0444	1.6465				
	1.8106	1.4942				
	1.5040	0.0173				
61	2.0345	0.6717				
	2.8769	1.4278				
62	3.4519	2.4190				
	3.2913	1.9620				

linear functions of the light time fraction) are less than for white light. As regards  $\tau'$ , about the same rate of change (again less than for *white*) is shown for all four colors, although the absolute values of  $\tau'$  differ. With  $F_{\max}$ , the rate of change is least for *R*, greater for *G*, *B*, *V* in that order.

### III

We may now consider the properties of the "cone" curve parameters as a function of wave-length. It is to be noted that *color per se* is not in question,

since the level of occurrence of the color threshold at critical fusion intensity produces no modification in the form of the curve, and its position on the curve can be experimentally pushed about by changing the light time fraction.

TABLE II

*Blue*

<i>F</i> per sec.	$t_L = 0.10$		$t_L = 0.90$	
	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$
2			$\bar{7}.3753$	$\bar{9}.6708$
4			$\bar{7}.6883$	$\bar{8}.3120$
6			$\bar{6}.0485$	$\bar{8}.3660$
8			$\bar{6}.3229$	$\bar{8}.7401$
10	$\bar{7}.4962$	$\bar{8}.7900$	$\bar{6}.6532$	$\bar{7}.0004$
11	$\bar{7}.7098$	$\bar{8}.7943$		
12	$\bar{7}.8841$	$\bar{7}.2315$	$\bar{5}.0753$	$\bar{7}.4318$
13	$\bar{6}.1150$	$\bar{7}.5385$		
14	$\bar{6}.3641$	$\bar{7}.7757$	$\bar{5}.8795$	$\bar{6}.1774$
15	$\bar{6}.5934$	$\bar{7}.9182$		
16	$\bar{6}.8195$	$\bar{6}.2166$	$\bar{4}.0383$	$\bar{6}.5917$
17	$\bar{5}.0732$	$\bar{6}.4222$		
18	$\bar{5}.3916$	$\bar{6}.7108$	$\bar{4}.4422$	$\bar{6}.7871$
19	$\bar{5}.5983$	$\bar{5}.0421$		
20	$\bar{5}.7928$	$\bar{5}.2569$	$\bar{4}.9997$	$\bar{5}.4108$
	$\bar{5}.7833$	$\bar{5}.0801$		
21	$\bar{5}.9883$	$\bar{5}.4901$	$\bar{3}.2018$	$\bar{5}.6869$
22	$\bar{4}.3061$	$\bar{5}.9131$	$\bar{3}.4692$	$\bar{5}.9095$
25	$\bar{4}.6127$	$\bar{4}.0380$	$\bar{3}.7200$	$\bar{4}.2662$
30	$\bar{4}.9243$	$\bar{4}.4593$	$\bar{3}.8775$	$\bar{4}.6074$
35	$\bar{3}.3201$	$\bar{4}.6139$	$\bar{2}.3981$	$\bar{4}.7279$
40	$\bar{3}.6825$	$\bar{3}.1473$	$\bar{2}.7818$	$\bar{3}.3942$
45	$\bar{2}.1080$	$\bar{3}.3120$	$\bar{1}.1868$	$\bar{3}.5706$
50	$\bar{2}.6935$	$\bar{2}.0347$	$\bar{1}.7873$	$\bar{2}.3444$
55	$\bar{1}.5904$	$\bar{1}.6136$	$\bar{1}.0355$	$\bar{1}.4930$
56			$\bar{1}.5066$	$\bar{0}.2067$
57			$\bar{2}.1880$	$\bar{0}.7459$
58	$0.3219$	$\bar{1}.8353$		
59	$0.7887$	$0.1629$		

When this is done the  $\sigma'_{\log t}$  parameter is not changed. We shall consider the color threshold indications subsequently.

The curious point is at once apparent that  $F_{\max.}$ , for a given  $t_L$  percentage, can be higher (with *violet, blue, green*) than for *white*. At the same time the abscissa of the inflection point (save for the *red*) is *less* than for *white*. The reality of this drop is easily demonstrated by determining at a given convenient  $F$  the critical flash intensity for white light, then dropping a filter, say *blue*, in the path of the beam. Thus at  $F = 30$ ,  $t_L = 0.50$ ,  $\log I_m$  *white* was found

to be in one test (10 observations) =  $\bar{1}.7879$ ; with the *blue* filter dropped in place, it was  $\bar{1}.0755$  with no correction made for the transmission of the *blue* filter.

TABLE III

*Green*

<i>F</i> per sec.	$s_L = 0.10$		$s_L = 0.90$	
	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$
2	$\bar{7}.9764$	$\bar{8}.4127$	$\bar{5}.0403$	$\bar{7}.6359$
4	$\bar{6}.1432$	$\bar{8}.7000$	$\bar{5}.3433$	$\bar{7}.8121$
6	$\bar{6}.4411$	$\bar{8}.9579$	$\bar{5}.6233$	$\bar{6}.1902$
8	$\bar{6}.8018$	$\bar{7}.1851$	$\bar{5}.9820$	$\bar{6}.2848$
10	$\bar{5}.1502$	$\bar{7}.3278$	$\bar{4}.3879$	$\bar{6}.9491$
11	$\bar{5}.3179$	$\bar{7}.7291$		
12	$\bar{5}.4692$	$\bar{7}.8702$	$\bar{4}.6436$	$\bar{5}.1241$
13	$\bar{5}.5740$	$\bar{7}.9442$		
14	$\bar{5}.6650$	$\bar{6}.1208$	$\bar{4}.8864$	$\bar{5}.3037$
15	$\bar{5}.8274$	$\bar{6}.2537$		
16	$\bar{5}.9837$	$\bar{6}.4403$	$\bar{3}.1637$	$\bar{5}.6732$
17	$\bar{4}.1144$	$\bar{6}.5515$		
18	$\bar{4}.2777$	$\bar{6}.8429$	$\bar{3}.4064$	$\bar{5}.7810$
19	$\bar{4}.3974$	$\bar{6}.8337$		
20	$\bar{4}.5325$	$\bar{6}.9507$	$\bar{3}.6880$	$\bar{4}.0562$
	$\bar{4}.5407$	$\bar{5}.3258$		
21	$\bar{4}.7025$	$\bar{5}.2040$	$\bar{3}.8818$	$\bar{4}.2889$
22	$\bar{3}.0179$	$\bar{5}.4773$	$\bar{2}.2416$	$\bar{4}.7579$
23	$\bar{3}.1641$	$\bar{5}.6069$	$\bar{2}.3669$	$\bar{4}.7182$
24	$\bar{3}.2899$	$\bar{5}.7127$		
25	$\bar{3}.3735$	$\bar{5}.7113$	$\bar{2}.5503$	$\bar{3}.0102$
30	$\bar{3}.6833$	$\bar{4}.1846$	$\bar{2}.8274$	$\bar{3}.2941$
	$\bar{3}.6573$	$\bar{4}.0748$		
35	$\bar{2}.0776$	$\bar{4}.6332$	$\bar{1}.1763$	$\bar{3}.5717$
40	$\bar{2}.4538$	$\bar{3}.0915$	$\bar{1}.5170$	$\bar{3}.8274$
	$\bar{2}.4347$	$\bar{3}.0736$		
45	$\bar{2}.8551$	$\bar{3}.1524$	$\bar{1}.9900$	$\bar{2}.3929$
50	$\bar{1}.4955$	$\bar{3}.7677$	$0.6729$	$\bar{1}.1175$
55	$0.3528$	$\bar{2}.8836$	$1.8432$	$0.3348$
57	$0.9117$	$\bar{1}.4890$		
58	$1.0787$	$\bar{1}.3467$	$3.1156$	$1.4797$
	$0.9956$	$\bar{1}.3752$		
60	$2.4767$	$\bar{1}.1170$		
	$1.8965$	$0.4859$		

The general lowering of  $\tau'$  and the increase of  $F_{\max.}$  correspond, of course, to the meaning of a considerable variety of facts relating to the action of colored lights which have been curiously suppressed in the discussion of color theory as involving the general problem of excitatory efficiency. We shall return to this in a following section. Here we need only comment on the fact that

this type of change is, in and of itself, consistent with the general lowering of the brightness level required for violet, blue, and green wave-lengths (as compared with red) along the  $F - \log I$  contour. We say "consistent with," because the same type of change is found for *white* light when  $t_L$  is decreased, and (as demonstrated in the present paper) for single color regions. The theory

TABLE IV

*Red*

$F$ per sec.	$t_L = 0.10$		$t_L = 0.90$	
	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$
2	5.5989	7.9728	4.6960	5.0689
4	5.8639	6.3329	4.9481	5.4192
6	4.1711	6.7534	5.2890	5.6591
8	4.5369	6.9153	5.6631	4.0521
10	4.9014	5.3582	5.0154	4.6341
12	5.1596	5.6409	5.3252	3.1796
14	5.4023	5.8330	5.5223	4.8714
16	5.6889	4.0843	5.8301	3.3010
18	5.9568	4.2745	1.0653	3.5427
			1.0635	3.5734
20	5.1833	4.6001	1.2888	3.5664
	5.1701	4.6978	1.2847	3.7944
25	5.5223	4.8714	1.6680	2.0358
30	5.8398	3.2684	1.9890	2.2521
35	1.1611	3.5850	0.2962	2.6459
40	1.5431	2.0760	0.6665	1.2359
	1.5407	2.0760		
	1.5407	3.8175		
45	1.8705	2.5166	1.0178	1.9375
	1.9768	2.4033		
	1.9742	2.4599		
50	0.5172	1.0531	1.8050	0.0451
	0.5192	2.9342		
52			1.9021	0.1959
55	1.2878	1.1166		
57	1.9008	0.1479		

of this increase of  $F_{\max}$ , as a result of decreasing  $t_L$  has been that the frequency of elementary contributions from excitable units has been increased (Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *a, b*; Crozier and Wolf, 1940; 1939-40 *b*). The increase of  $F_{\max}$ , could thus be produced, other things equal, by using light of intrinsically lower excitatory power, and this must be expected to automatically lower  $r'$ .

This general situation cannot be reconciled in terms of photometric brightness, because of the data with *red* light and because there is no correction

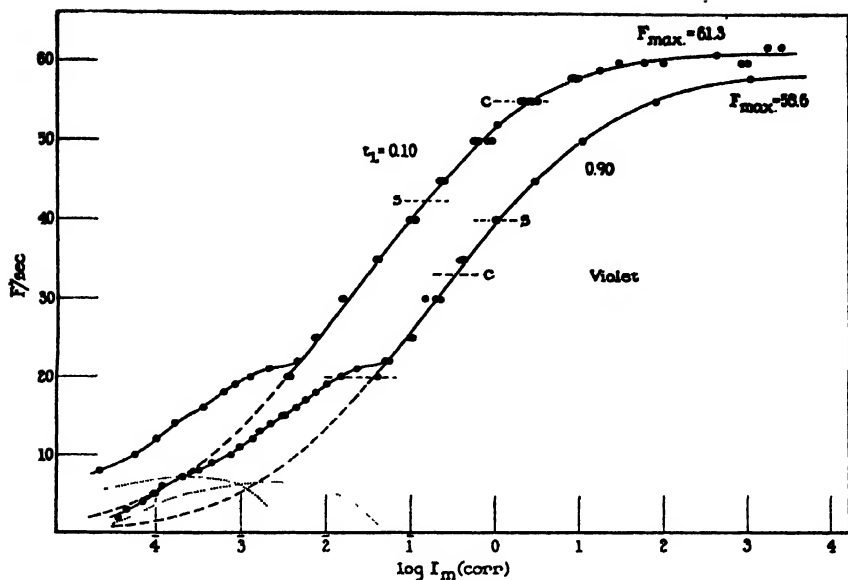


FIG. 2. Figs. 2, 3, 4, and 5 give  $\log I_m$  as a function of  $F$  for the *violet*, *blue*, *green*, and *red* respectively, for light time fractions  $t_L = 0.10$  and  $0.90$ . Data in Tables I to IV.

On each curve is marked the position of the color threshold ( $C$ ) and the level above which the critically fused field is smooth ( $S$ ).

An exceptional series with *violet* is shown in Fig. 6; it ties in with the aberrant point at the "corner" of the curve here given.

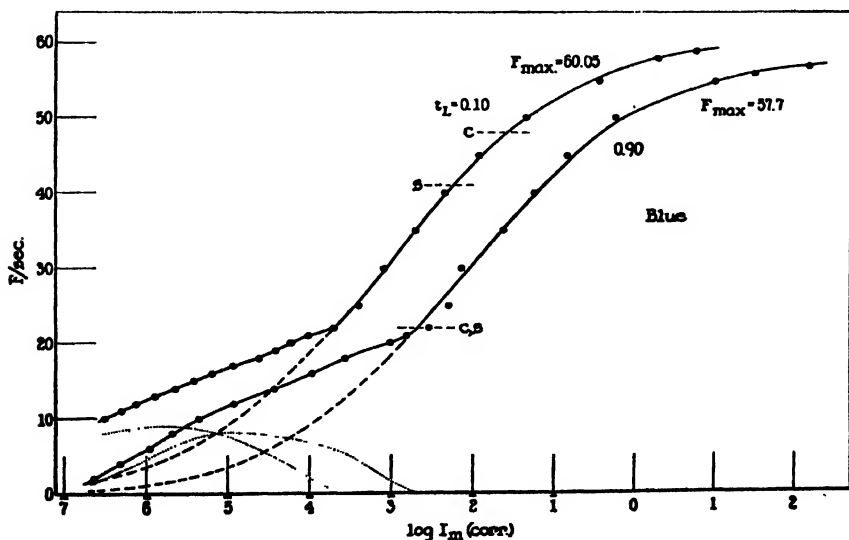


FIG. 3. Blue



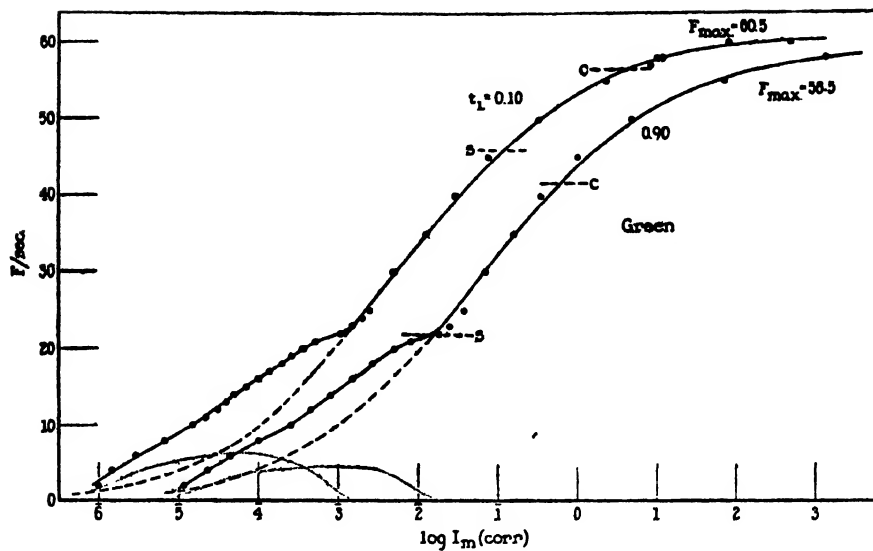


FIG. 4. Green

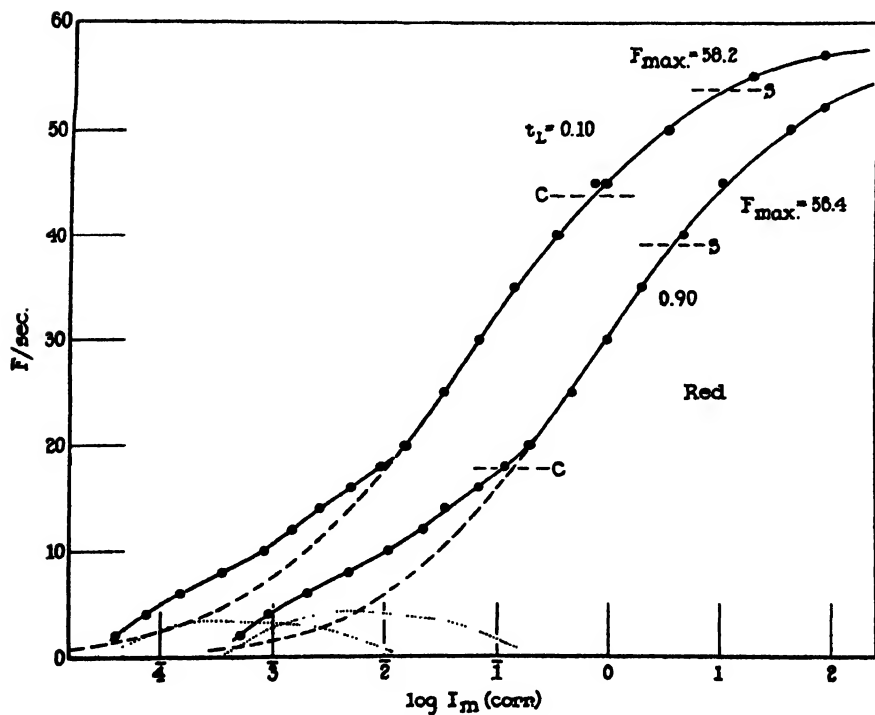


FIG. 5. Red

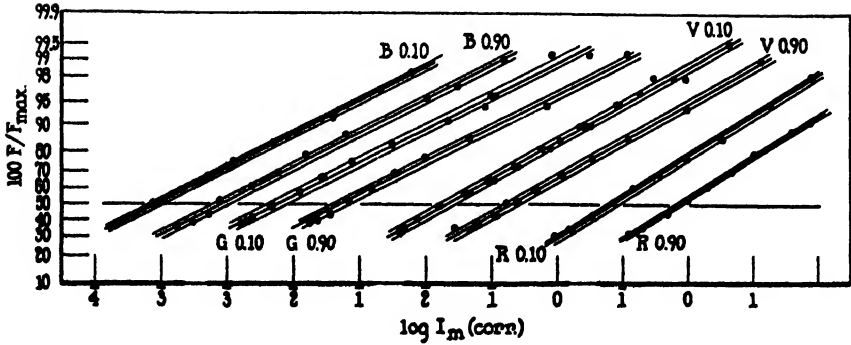


FIG. 6. The upper segments of the data in Figs. 2 to 5, on a probability summation grid.

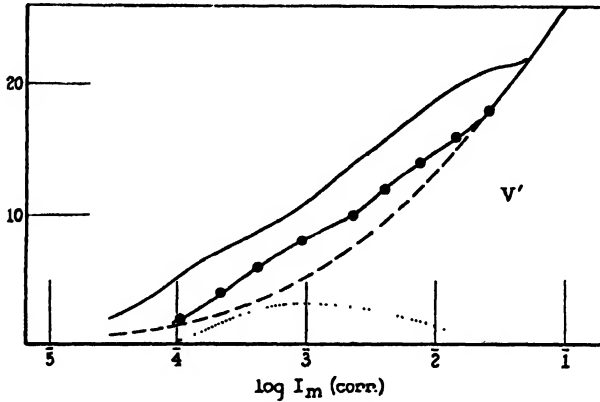


FIG. 7. An exceptional series of measurements with *violet*; see text; the curve of Fig. 2 is also given.

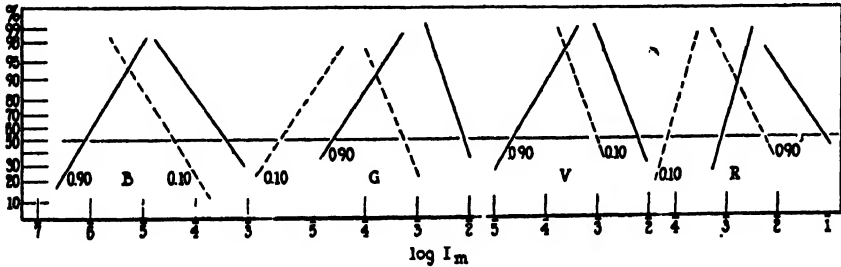


FIG. 8. The isolated "rod" contribution curves (Figs. 2 to 5) with their rising and falling branches transferred to a probability grid.

possible in terms of the "visibility function" for the several spectral regions we have used. We are convinced that a distinctly different mode of approach must be exploited before a valid and fruitful connection can be detected between the data of flicker and some other data involving the problem of visual excitability as a function of wave-length. We are also of the opinion that, significant as such a connection must be for hypotheses of color vision, it has been unnecessarily ignored—although we do not need to detail at this point our reasons for the statement.

The theoretical analysis of flicker response contours has shown for the simplex  $F - \log I_m$  curves of invertebrates and vertebrates and for the data

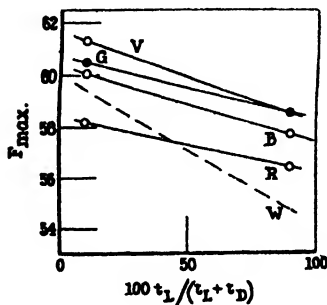


FIG. 9

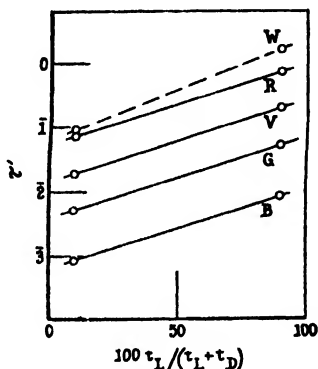


FIG. 10

FIG. 9. The relation between  $F_{max.}$  and the light time percentage, for the four colors used, and for white.

FIG. 10. The change of the "cone" abscissa of inflection ( $\tau'$ ) in the  $F - \log I_m$  curve as a function of the light time percentage.

on vertebrates (including man), that the magnitudes of  $F_{max.}$  are in direct proportion to the percentage dark time in the flash cycle (white light), while  $\tau'$  is inversely proportional to this percentage (Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *a, b*; Crozier and Wolf, 1939-40 *b*). The question therefore arises, as to how it is possible to obtain a larger total number of potentially effective elements ( $\propto F_{max.}$ ) by using the blue light, or violet, or green, filtered from a white, than by using the unfiltered white. There are two general ways in which this question could be approached. It could be supposed that different regions of the spectrum have "antagonistic" effects when combined in white. Qualitatively, a number of different kinds of not very satisfactory instances could be brought in support of such an idea (*cf.* Wilson, 1891; Babák, 1913; but *cf.* Crofts and Laurens, 1924; Polimanti, 1916; *etc.*; and observations by Crozier and Cole, 1924, on the circus movements of *Limax*, as yet unpublished). It does not necessarily amount to a denial of this general conception

to insist that some further considerations must first of all be dealt with. These latter considerations arise because of the basic assumptions required by the properties of the  $F - \log I$  contour. The properties in question are of two kinds: The nature of the analytical function describing this contour, and the known quantitative attributes of its parameters.

The form of the function as a normal probability summation in  $\log I$  is deduced on the basis that the neural units excited fluctuate in their momentary thresholds and in their momentary contributions to the determination of the observable result. For the examination of excitation as a function of wave-length of light, and for the estimation of excitabilities in different parts of the retina, means must be found for the separation of these two contributions—the number of units, and the frequency distributions of their contributions of elements of effect.

The independent properties of the parameters of the function—that is, of its asymptotic maximum ( $F_{\max}$ ), of its abscissa of inflection ( $\tau'$ ), and of the standard deviation of its first derivative ( $\sigma'_{\log I}$ ), demonstrate objectively that the situation is essentially *multivariate*; a situation, that is, in which the quantitative relationship to be found between any two variables is, in the last analysis, dependent on the prevailing magnitudes of the other variables operating (Crozier 1939 *b*; 1940 *a, b*). It thus becomes impracticable to establish in advance of quantitative information anything in the nature of “standard conditions” for the comparison of the wave-length effects. In the case of flicker, one cannot be assured that  $\tau'$  or  $F_{\max}$  will exhibit the same kind of dependence on retinal area illuminated, retinal location of test-image, light-time fraction, or temperature of the organism, for lights of different wave-length compositions. In fact, so far as tested they do not.

One thing can be indicated, however. With *white* and with lights of different colors the parameter  $\sigma'_{\log I}$ , the standard deviation of  $dF/d \log I$  with  $F_{\max}$  put = 100, is (for each wave-length composition) independent of the light-time fraction (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *a, b*; Crozier and Wolf, 1939–1940 *b*, and Fig. 8). In the experiments where  $t_L$  is varied the assumption has been that the basic excitability of the neural units concerned is not affected, but merely the frequencies with which they contribute elementary neural effects. One proof of this, and an entirely adequate proof, is found in the fact that  $F_{\max}$  is independent of temperature, and the temperature characteristics for excitability are independent of the light time fraction (Crozier and Wolf, 1939–40 *b*) and of the form of the  $F - \log I$  curve (*cf.* Crozier 1939 *a*; Crozier and Wolf, 1939–40 *a*). Consequently we may legitimately compare  $\sigma'_{\log I}$  for the various regions of the spectrum. Indication should thereby be given as to the relative numbers of units involved, quite apart from the question as to the frequencies of their contribution to the determination of the measured result. The basis for this expectation is found in

the now known results obtained by deliberately varying the numbers of these units in other ways. This number may be changed by altering the area of the test-field (*cf.* Piéron, 1935; Hecht and Smith, 1935-36), by placing it in different parts of the retina, and by changing the level of light adaptation from which dark adaptation is measured (*cf.* Crozier, 1940 *b*). Speaking of cone units, it is found that increasing the number of cone units brings about (above a certain small number) a *decrease* of  $\sigma'_{\log I}$  (Crozier, 1940 *a*); whereas, when the "rod" function can be followed without thus far detected cone complication, increasing the effective number of rod units brings about an *increase* of their  $\sigma'_{\log I}$  (Crozier, 1940 *b*).

At the moment we deal only with the "cone" branch of the duplex flicker curve; the "rod" portion must for various reasons be considered separately. We find (Fig. 6) that the "cone"  $\sigma'_{\log I}$ , although independent of  $t_L$ , is definitely a function of the wave-length composition. We have data showing that for the several colored lights used in the present observations the general rule here referred to is obeyed when comparison is made with tests in a region of the retina where the cone density is smaller (*cf.* Crozier and Wolf, 1941-42). In the present measurements (Fig. 8),  $\sigma'_{\log I}$  is least for *red*, approximately the same for *green* and *blue*, greatest for the *violet*. Although the differences are not tremendous, they are abundantly real. It is significant that the rates of change of  $F_{\max.}$  with  $t_L$  are (as already noted—Fig. 9) in just this order. The point has been made before in a general way that the shapes of the  $F - \log I$  curves for different wave-lengths do not differ greatly (Hecht and Verrijp, 1933; Hecht, Schlaer, and Smith, 1935; Hecht and Schlaer, 1935-36; Hecht, 1937). The fact is, however, that form differences such as are here emphasized are contained in these older data as well, although not necessarily in the same order. In terms of the general proposition already stated, the *violet* here involves the smallest, the *red* the largest number of cone units, and the *green* and the *blue* about the same intermediate number. The respective values of  $F_{\max.}$  are in the general declining order (Fig. 9): Violet, green, blue, red, and consequently there is a real qualitative agreement between the indications provided by these two parameters. The indication is that the smaller number of "cone" units provides the largest number of elements of effect. For our *white*,  $\sigma'_{\log I}$  is in between the values for *blue* and *red*; the values of its  $F_{\max.}$ , although intermediate, cannot be directly compared because they have quantitatively a different relation to the light time fraction.

Such considerations do not in many respects agree with some traditional views of the basis of color ( $\lambda$ ) excitation. It might, of course, be sought to ignore the problem presented by supposing that the data of flicker can give no information about visual excitation as a function of wave-length. Perhaps some of the background for such an opinion could be found in vague general impressions as to the basis for flicker photometry—a distinctly different matter.

But since differences of the general kind here stressed are certainly valid, as comparisons with earlier observations show (Ives, 1912, 1922, *etc.*), it would be patently idiotic to deny their possible utility. The import they convey is that the number of ("cone") neural units excited by *red* is more than for *violet*, while the *green* and *blue* are intermediate in this respect, like *white*. And at the same time it is clear that the slight differences between the shapes of the flicker color curves are not inconsistent with the more modern view (Hecht, 1931, 1932, 1934) according to which color vision data can be described in terms of the spectral sensibility distributions of (three) species of [retinal] cones, of approximately equal numbers, and of approximately the same spread of wave-length sensibility. The hitch in this general view is in the definition of "sensitivity"; from the flicker data it can be assumed that the numbers of responsive cones in the three primary classes are about equal, or at least not very unequal: but the elements of neural effect they produce differ in number.

Disregarding for the present the possibility that different regions of the spectrum may in *white* "act antagonistically," for which special tests can be devised, and which is certainly not inconsistent with the present data, it is obvious that the facts thus far presented have no simple relation to traditional hypotheses of color vision. It cannot be asserted that *red* for example, or *blue*, acts simply on one-third or one-quarter of the "cone" units, if the white acts on all of them. The discussion of this problem is usually undertaken in distinctly different terms, namely with reference to the intensity required for excitation, for example at the visual threshold. The question then becomes one of reducing the flicker data to a valid basis for the comparison of wave-length effects. Comparisons cannot be made at fixed values of  $F$  or of  $I_m$ , since the forms of the curves are not the same. We can, however, eliminate some of the complication due to the difference between number of *units* activated and number of *elements* of effect produced by finding (graphically from Figs. 9 and 10) the values of  $t_L$  which reduce the color curves to the same  $F_{max}$ . (this does not change  $\sigma'_{log I}$ ); from the graphs the corresponding values of the abscissae of inflection  $\tau'$  are then read. From these values of the flash intensity  $I$  and of  $t_L$  the mean relative photometric energy flux is obtained for activation of 50 per cent of the flicker recognition elements, as a function of the spectral region, under such conditions that each spectral region can activate the same total number of elements. The data for a comparison of this kind are given in Table V.

The calculation is necessarily rough. For one thing, the slope of the  $\tau'$  (and  $F_{max}$ .) *vs.*  $t_L$  function is higher for *white* than for the colors, so that the result depends somewhat upon the level of  $F_{max}$ . chosen. Moreover, the colored lights used are not monochromatic. Nevertheless it is apparent that, in terms of flash intensity as estimated by dim light visual photometry against white, the maximum of effectiveness is in the blue-green, the minimum in the

violet. When (Table V) the intensities are corrected to an energy basis by means of the thermopile measurements, the *mean* value of  $\tau'$  (for the fixed  $F_{\max.}$ ) for the 4 colors (2.58) is in good agreement with that for white (2.60).

It is not at all impossible to employ this method of analysis under conditions of greater refinement as to  $\lambda$ . The customary method of procedure compares threshold intensities under fixed conditions. The present general method deals with the intensities required to produce proportionately equivalent effects as measured. It thus avoids the impossible assumption that threshold effects are "constant" and "equal" (*cf.* Crozier, 1939b; 1940a). From data on  $\Delta I_0$  as a function of exposure time it would be possible to estimate (*cf.* Crozier, 1940a), with area fixed, the value of  $\log t_{\exp.}$  required for 50 per cent activation, as a function of  $\lambda$ . Such estimates would, for this area and retinal position, give basis for a valid visibility curve. Similarly, the analysis of differential

TABLE V

From interpolated values (Figs. 9 and 10) of  $t_L/(t_L + t_D)$  the levels of  $\tau'$  are estimated which are connected with  $F_{\max.} = 58.5/\text{sec.}$ , for four spectral regions and white. The mean energy flux (with flash intensity in photometric units) critical for flicker is then given at this point of 50 per cent activation in the corresponding "cone" populations of elements, and from thermopile readings in the last column.

	$t_L$ for $F_{\max.} = 58.5$	$\tau'$	Log relative mean flux	With thermopile correction
Violet.....	91	1.31	1.27	2.76
Blue.....	69.5	3.66	3.50	2.39
Green.....	87.8	2.72	2.66	2.94
Red.....	37	2.63	2.20	2.23
White.....	25	1.20	2.60	2.60

intensive excitability ( $1/\Delta I$ ) as a function of  $\lambda$  and of  $I_1$  (*cf.* Crozier, 1940c) can be made in precisely the same manner. Only in this way, it appears, can invariant indices be discovered making possible the real interpretation of the influence of such other variables as, for example, oxygen pressure.

The rôle of the intensity at fusion can be rather directly seen in the behavior of the color thresholds. In Figs. 2 to 5 the flash intensities at the appearance of color on the  $2F - \log I$  curves are for any one color separated by  $0.86 \pm 0.11$  log unit; since the mean flux along the two curves ( $t_L = 0.10$  and  $0.90$ ) differs by  $0.95$  log unit, and since observations were not made continuously along the curve but at comparatively coarse intervals, the agreement is taken to be close. Similar considerations hold for other subjective phenomena along these contours (*cf.* section V).

## IV

The appearance of the raw "rod" curves in Figs. 2 to 5 is in a general way similar to that already known (*cf.* Hecht and Shlaer, 1935-36). For the same

test area and light time fraction the *white* curve rises more abruptly and crosses the lines for the colored lights, when the "cone" curves are brought together by putting their inflection points at the same flash intensity (Fig. 11). The *blue* curve is more nearly horizontal than the others, the *red* steepest; *violet* and *green* are intermediate and do not differ greatly.

When the "rod" contribution is dissected out by the method previously illustrated it is found that  $\sigma'_{\log I}$  for the ascending curves is, as nearly as can

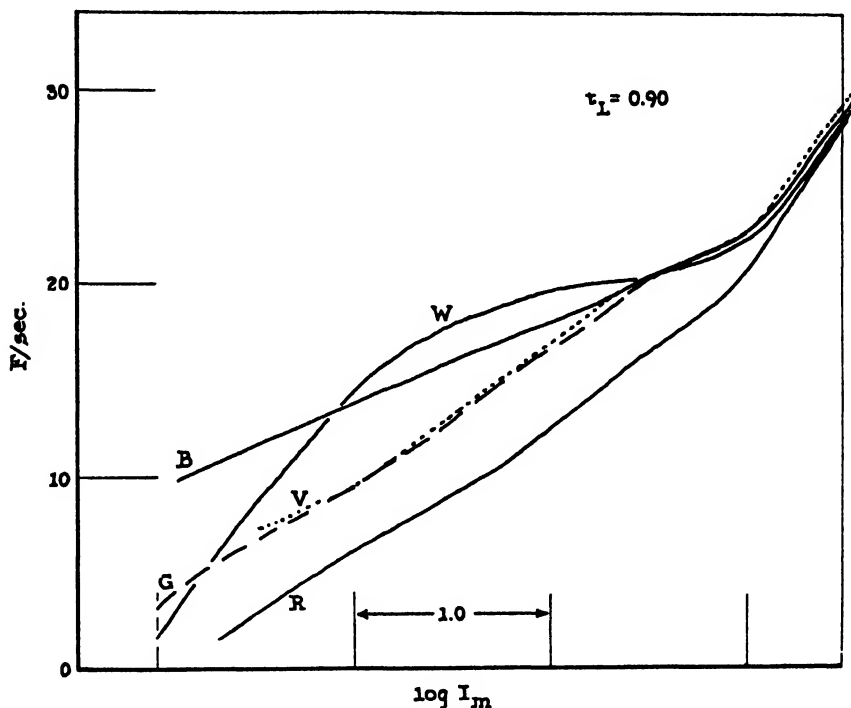


FIG. 11. The comparative slopes of the lower segments of the  $F - \log I_m$  contours for white (Crozier and Wolf, 1940-41b) and four colored lights, at  $t_L = 0.90$ , the cone abscissa of inflection being brought to the same  $\log I$ .

be told, independent of  $t_L$  (as for white) and of the (apparent) "rod"  $F_{\max}$ . for each color. It is greatest for the *green*, intermediate for *blue* and *violet*, least for *red*. The *red*  $\sigma'_{\log I}$  is less than for any of the others, indicating a smaller population of (rod) excitable units, but this is not necessarily true for other parts of the retina. This order differs from that with the "cone" curves (section III) in the relatively *larger* fundamental population of units thus indicated for the *green*, and *smaller* for *red*. It has already been shown (Crozier and Wolf, 1940-41b) that the form of the *declining* "rod" curves must be understood as due to the progressive inhibition of "rod" elements, and that the



value of the "rod"  $F_{\max}$  is governed in the same way. We might then expect that a certain direct correlation should obtain between the apparent  $F_{\max}$  and the log  $I$  separation of the inflection points of the "rod" and "cone" summations. For any one value of  $t_L$  it is clear that (except for *white*) this rule is obeyed: thus at  $t_L = 0.10$  we have:

	Computed "rod" $F_{\max}$	( $r'_{\text{cone}} - r'_{\text{rod}}$ )
<i>Violet</i> .....	6.4	3.94
<i>Blue</i> .....	8.2	4.02
<i>Green</i> .....	4.4	3.35
<i>Red</i> .....	4.2	2.97

With fuller information it might be possible to derive the unhampered form of the "rod" curve as a function of wave-length, but this cannot now be attempted. We can rely only on the suggestion obtained from the  $\sigma'_{\log I}$  indices.

In speaking of the separation of "rod" and "cone" effects it is necessary to point out that, as with white light (Crozier and Wolf, 1940-41b), the indications provided by different criteria for the incidence of "cone" effects do not give an entirely consistent picture. The kink in the  $F - \log I$  contour has commonly been taken to give a dividing point between rod and cone function; under certain conditions (white light, and the usual  $t_L = 0.50$ ) this point coincides pretty well with the transition to a "smooth" appearance of the field as critically adjusted for fusion and the change of the character of the "Flimmern" end-point to a fine-lined flicker. With other light time fractions this correlation is upset. So also with *color* at the flicker fusion end-point. Careful note was made during the experiments as to the subjective appearance of the critically fused field at the points plotted along the curves and at various places in between. The color thresholds as observed in this way are marked on the graphs, as well as the levels at which, progressing upward, the field assumed a smooth appearance (*i.e.*, no longer granular or "frosted").

From these records it is evident that, with *violet*, *blue*, and *green*,  $t_L = 0.10$ , the point of appearance of the proper color is rather high up on the curve. With *red* it is relatively a little lower. Using  $t_L = 0.90$ , however, the color point is pushed down close to the bend for *blue* and *red* although still far above it with *violet* and *green*. The smooth type of field-at-fusion may appear above or below the color level, in different cases. A certain amount of fluctuation is found in the occurrence of these points, and this is easily influenced by fatigue. With *violet*, *blue*, and *green* the contrast after image color is detectable at critical flash-frequencies below the color point. We are convinced that the systematic examination of these matters can be rewarding, but at present it need only be emphasized that the criteria of separation of cone function as obtained from the shape of the curve have no necessary, simple relationship to the others mentioned. Similarly, the smooth "blue-gray" character of the field

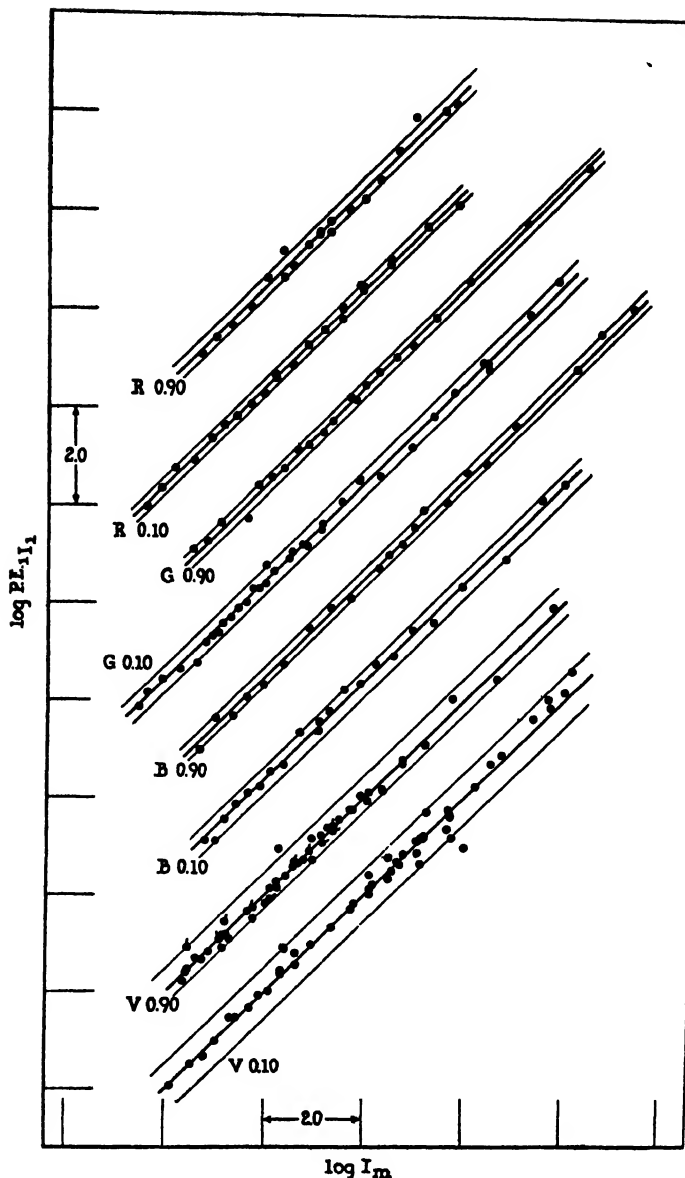


FIG. 12. The relation between  $I_m$  and  $P. E._{1I_1}$  (Tables I-IV), for the two sets of measurements ( $t_L = 0.10, 0.90$ ) with *violet, blue, green, and red*. On a log-log grid the points form bands with slope = 1 and of statistically determinate width, related to  $t_L$  and to wave-length ( $F_{max.}$ ); see text. (Since we are interested only in the relative variation, the bands have been separated for clearness.) For V, the aberrant series depicted in Fig. 7 is here given with tags.

along the "rod" curve may (*violet, red*,  $t_L = 0.10$ ) persist rather far up the "cone" branch or (all the colors,  $t_L = 0.90$ ) it may give way to a speckled, granular field at half the distance up the "rod" curve.

## V

The uniocular flicker response measurements with white light have shown that the *mean* proportionality constant for the rectilinear relationship between  $I_m$  and the dispersion of  $I_1$  is independent of the light time fraction (Crozier and Wolf, 1940-41b). At the same time, it is not independent of organic, central nervous factors; it is reduced by binocular regard in the ratio of  $\sqrt{2}$ :1 (Crozier and Wolf, 1940-41a). For a given value of  $I_m$ , consequently, independently of  $F$ , the associated variation of  $I_1$  is independent of the total population of potentially excitable elements ( $\propto F_m$ ), but is a function of the potency which these elements exercise in effecting the statistical discrimination of light from darkness in the flicker cycle. Fig. 12 shows, when the corresponding proportionality constants are evaluated, that there is no relation between this factor and wave-length composition, because it is constant. This agrees with what we know (Crozier and Holway: in preparation) on the basis of  $\Delta I$  determinations with colored lights.

There is, however, a definite correlation between the *scatter* of the dispersion indices and  $F_{max.}$ , both as regards the effect of the light time fraction and of the wave-length composition (*violet, blue, green, red*). This is crudely visible in Fig. 12. It is easily stated more exactly by giving the mean and the S. D. of the distribution of the proportionality constants (*cf.* Crozier and Holway, 1937; Crozier and Wolf, 1940-41a). As the total size of the potentially available group of (cone) units is increased, the precision with which  $\sigma_{I_1}$  (and thus  $I_m$ ) is determined is definitely diminished. This corresponds to the fact that with *white* (Crozier, 1940) increase of size of the cone population of units (and thus of the elements of effect they produce) increases the dispersion of their effectiveness (denoting "interaction"). The spread constants of bands in Fig. 12 follow this rule, except for *red*  $t_L = 0.90$ , where two points only are aberrant; the value for *violet*  $t_L = 0.10$  is also rather high. Sporadic occurrence of irregular cases of this kind is, of course, to be expected on grounds of statistical sampling: For *green* and *blue* the spread constants, at a given  $F_{max.}$ , are less than for white. Highly homogeneous data would be required for the further development of the possibilities here suggested.

## VI

## SUMMARY

For spectral regions associated with violet, blue, green, and red the relation between mean critical flash intensity  $I_m$  for visual flicker and the flash frequency

$F$  is modified as already found with white light when the light time fraction  $t_L$  in the flash cycle is changed. For a square image  $6.13^\circ$  on a side, foveally fixated, the "rod" and "cone" contributions to the duplex contour are analyzed in the way already used for white. It is pointed out that several customary qualitative criteria for cone functioning do not necessarily give concordant results. The analysis shows that the three parameters of the probability summations giving the "rod" and "cone" curves are changed independently as a function of wave-length composition of the light, and of the light time fraction.

The correlation of these changes, and of those found in the associated variability functions, can be understood in terms of differences in (1) the numbers of neural units potentially excitable and (2) in the numbers of elements of neural effect obtained from them. In a multivariate situation of this kind it is necessary to compare intensities of luminous flux required to activate half the total population of potentially available elements when this total size is held constant for the different conditions. The results of this comparison, for the filtered lights used, are discussed in relation to certain aspects of excitation *vs.* wave-length. The problem is a general one, arising where the effects produced as a function of a particular variable are concerned. In the distinction between (1) units excited and (2) the actions they produce may be found the clue for the curious fact that with certain wave-lengths the critical intensities are lower than for white. The extension of the observations to other parts of the retina may be expected to further this analysis.

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# X-RAY AND CRYSTALLOGRAPHIC STUDIES OF PLANT VIRUS PREPARATIONS

## I. INTRODUCTION AND PREPARATION OF SPECIMENS

## II. MODES OF AGGREGATION OF THE VIRUS PARTICLES

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PLATES 1 TO 4

(Received for publication, March 14, 1941)

### INTRODUCTION

Since their original isolation by Stanley in 1935, the protein preparations from plants suffering from virus diseases have been much studied, but chiefly chemically and biologically. This paper is an account of a physical and crystallographic study of virus preparations which was carried out in conjunction with the work of Bawden and Pirie (1937 *a, b*; 1938 *a, b, c*).

The interest of this study is twofold, physical and biological. The properties of the virus preparations, particularly those that exhibit liquid crystal character, show many novel phenomena that throw much light on physicochemical problems, especially on those of colloid systems. They have revealed unsuspected regularities in the arrangement of long particles in anisotropic solutions, and thrown light on the structure of the various forms such liquids exhibit. The study of these substances has necessitated the development of new techniques of x-ray analysis and has resulted in the extension of their use to the measurement of repeat distances of the order of hundreds of Ångström units, thus going far to bridge the gap between x-ray and microscopic observations.

The properties revealed by these methods are exhibited in perfection by the virus preparations, but there is no reason to believe that they are confined to them, since they might occur in any aggregate of similar and markedly anisotropic particles. But x-ray analysis has revealed internal structures which are characteristic of the virus particles themselves.<sup>1</sup> This is shown by their

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<sup>1</sup> We have designated throughout the particles found in virus preparations as virus particles without prejudice to the question of whether they are or are not the infective agent. This can only be answered by biological means but already the weight of evidence seems to indicate that they are: (see Bawden and Pirie, 1937 *a, b*; 1938 *a, b*).

relative independence of the state of aggregation and by significant differences between different strains and species of virus. The virus particles have been shown to possess a regular internal structure which can be partially interpreted. These structures show interesting similarities and differences from those of both crystalline and fibrous proteins and help in turn to throw light on their constitution. The x-ray and optical studies are complementary to the biological investigations of the virus and give a more concrete picture of the different states in which it exists both in nature and in artificial preparations. Finally, the comparative study of these structures shows an interesting parallelism with their biological relationships.

### *The Progress of the Investigation*

In giving a rational account of all these somewhat complex phenomena, it is necessary to depart widely from the historical order of discovery. It may therefore not be out of place to preface it with a short account of the actual steps of the work which will also serve as an introduction to all the main phenomena observed.

In 1935 Stanley isolated from tobacco and tomato plants suffering from mosaic disease substances which he described as crystalline and which appeared to possess the properties of the virus. Shortly afterwards, Bawden and Pirie, using different methods of preparation, obtained solutions which separated into two layers, the lower of which exhibited a marked sheen. It was at this stage that we first examined the preparations and observed the spontaneous birefringence of the bottom layer solution and the flow birefringence of the top layer solution. The next stage was the production from these, by drying, of oriented preparations, the so called "wet" and "dry" gels.

An x-ray examination of the dry gel showed a fibre diagram similar to that of many proteins. It also showed what appeared to be strong equatorial reflections at very low angles. X-ray apparatus was developed to study these in detail and this revealed a pattern of five reflections corresponding to a hexagonal close packing of long particles with a diameter of approximately 150 Å.

At this point the x-ray investigation diverged into two lines. One dealt with the high-angle scattering and consequently with the internal structure of the particles, and the other with the low-angle scattering and consequently largely, though not entirely, with their mutual arrangement. These investigations are referred to subsequently as "intramolecular" and "intermolecular" respectively.

It was early shown that the intramolecular x-ray pattern was independent of the state of aggregation; dry and wet gels, bottom and even top solutions giving approximately the same pattern. This showed the persistence of a definite unit which may be properly called the virus particle.

The intermolecular pattern, however, showed a complete and quantitative dependence on the state of aggregation. All preparations, except the top solution, showed a regular hexagonal arrangement of particles, in which the particle distance varied

with the concentration. This showed that even in weak solutions, forces were acting between the particles capable of preserving a parallel and equidistant arrangement. An important negative result was that no indication of the length of the particles could be found by x-rays (Bawden, Pirie, Bernal, and Fankuchen, 1936).

It was next shown that the "crystals" originally described by Stanley (1935, 1937), were identical with the wet gel preparations and possessed therefore regularity only in two dimensions and not in three as in true crystals. It could then be said that all but one of the phases of the virus had substantially the same structure. The one exception was that of the crystals found in some infected plants which were too small for x-ray examination but appeared to be true crystals confirming the idea that the virus existed in a different form in the plant from that of any of its preparations studied by us. (However, see the paper by Loring, Laufer, and Stanley, 1938.)

Examinations of the intra- and intermolecular patterns of different strains of tobacco mosaic virus, cucumber viruses 3 and 4, and potato virus X (Bawden and Pirie, 1937 *b*, 1938 *a*) were also made and showed significant and illuminating similarities and differences (Bernal and Fankuchen, 1937). Subsequent observations have clarified and to a certain extent corrected these findings. An unexpected discovery was that of the single crystal character and the secondary orientation relative to the water surface of the gels formed by drying. This orientation is not shown by the particles themselves, demonstrating even more clearly the independence of the internal structure and the external arrangement of the virus particles.

More detailed investigation of the inner structure of the particles brought to light a high degree of regularity and complexity. The most perfect results were given by solutions, showing that the internal regularity is a characteristic of the virus particle itself (Bernal, 1938). The complexity of the x-ray pattern for long baffled interpretation until it was discovered that it was due to the presence of intensity maxima not conformable with Bragg's law owing to the small number of cooperating planes. It was then possible to establish a hexagonal unit cell ( $a = 87 \text{ \AA}$ ,  $c = 68 \text{ \AA}$ ) and to say something of the internal structure and its resemblances to and differences from those of the crystalline proteins that have been studied by x-rays.

A most fundamental simplification resulted from further study of gels of different water contents and also of those swelled in acid and salt solutions. This showed conclusively that all degrees of interparticle distance were possible and that there was no fundamental distinction between dry gels, wet gels, and bottom solution. These are all one phase with "top layer" solution as the only other phase. It further showed that the particles did not swell on wetting as had been thought at an intermediate stage of the investigation, and that their distance apart was entirely due to the properties of the solution in which they were immersed. A knowledge of this at once made apparent the explanation of the special physical properties of the virus—separation into layers, tactoid formation, etc. Indeed the theory can be further generalised to explain many colloid phenomena, such as thixotropy, and the structure of gels. A very promising explanation has been furnished by the theory of Levine (1939, *a*, *b*, *c*), which accounts for the interparticle forces as due to interpenetration of ionic atmospheres. Finally, one serious gap in the investigation of the virus, namely the question of the length of the particle, has probably been settled by the remarkable achievement of Kausche and coworkers (Kausche *et al.*, 1939), who have succeeded in photograph-



ing isolated virus particles in the electron microscope. Their observations are compatible with the picture of a thin straight particle of  $15\text{ m}\mu$  thickness, but show that the length is always a multiple of some unit of minimum length of the order of ten times the thickness, lengths beyond the range of observation of the x-ray methods used here.

All these results can be fitted into a general picture in which, however, only the outlines can be claimed to be well established. The views presented here must be taken as superseding in the light of fuller evidence those put forward in our earlier publications.

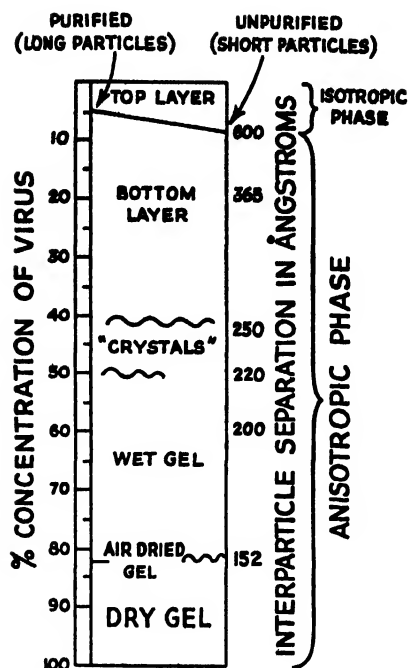
In what follows an attempt is made to achieve a logical order of presentation. In Part I a general description is given of the different phases of the virus preparations and of the methods used for examining them. In Part II the mutual arrangements of the particles in the different phases is discussed as well as the conditions controlling the relations between them. In Part III, Section 1, the evidence for their internal structure is presented; the relation of this to the structure of proteins is considered and on the basis of this, certain suggestions for possible structures are put forward. In the second section of Part III, comparisons are made between the structures of different strains and kinds of virus, and the possible biological implications of the physical investigations are discussed.

### *Preparation and Description*

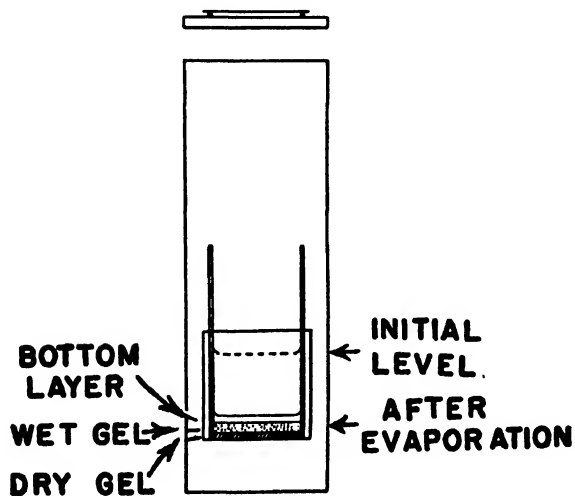
The purified virus preparations examined were (a) Tobacco mosaic virus, (b) enation mosaic virus, (c) aucuba mosaic virus, (d) cucumber virus 3, (e) cucumber virus 4, (f) potato virus X strain P, (g) potato virus X strain G, and (h) tomato bushy stunt virus. All these were prepared for us by Bawden and Pirie. For their methods the reader is referred to the original papers (Bawden and Pirie 1937 *a, b*; 1938 *a, b*).

We received the material except for the bushy stunt virus in the form of liquid crystalline solutions, containing from 5 to 34 per cent of dry weight virus. These several substances were examined in very different degrees of detail. The tobacco mosaic virus, which was not only the easiest to obtain but the stablest preparation, was most studied. In what follows all descriptions will be supposed to refer to it or to its very similar strains unless otherwise specified.

The properties of the virus preparations depend primarily on the concentration of virus. There are only two distinct phases, the disordered, and the ordered. The first is referred to as top layer solution and is an opalescent isotropic sol. The second varies from an anisotropic sol, referred to as bottom layer solution, through various stages of viscosity and rigidity to wet and finally to dry gel. Though all these form one phase and are not as was previously thought separate phases, it is convenient to treat them separately as they require different methods of preparation and examination. Top layer solutions are found for dilutions containing less than 4 per cent dry matter in the less well purified (and also probably less well aggregated) preparations,



TEXT-FIG. 1. General scheme of virus preparations.



TEXT-FIG. 2. Cell for making oriented dry gel.

or less than 1.8 per cent in the purest preparations. In concentrations greater than 1.8 per cent or 8 per cent according to the degree of purity and less than 34 per cent, the solutions referred to as "bottom layer," are spontaneously birefringent (Text-fig. 1). The more concentrated of these solutions were prepared by centrifugation (Bawden and Pirie, 1937 *a*). Bawden and Pirie describe them as jellies. They show, however, complete continuity with the more dilute solutions, differing only in their greater viscosity and we accordingly consider them to be part of the same phase as the bottom layer solution.

### *Preparation of Wet and Dry Gel*

The solutions on drying in ordinary vessels form a skin on the surface, which was early found to be birefringent and therefore showed that the particles were oriented relative to the air-water interface. This property was made use of to prepare oriented specimens of wet and dry gels by controlled evaporation in a simple cell. A drop of virus solution is introduced between a microscope slide and a cover-glass, kept apart by two glass threads, which can be of various thicknesses, according to the amount of material and thickness of gel required. The slide is then gently tilted so that the liquid fills the space between the glass rods down to the lower edge of the cover-slip (Text-fig. 2, and Plate 1 Fig. 1). The upper surface of the liquid being relatively protected from evaporation by the narrow space between the cover-slip and the slide does not form a skin. Most of the evaporation accordingly takes place through the skin at the bottom on which the solid material is deposited forming ultimately a layer of wet gel consisting of a single "crystal." The process is gradual and can be followed in a microscope. At an intermediate stage it could be seen from the birefringence that the concentration of the solution increased up to the sharp boundary of the wet gel where it attains the limiting birefringence of about 0.001. The wet gel forms a layer sharply distinguishable from this with a birefringence of 0.006. This distinction seems, however, simply to be due to the speed of drying and the inability of the more viscous concentrated solution to diffuse into equilibrium. The consistency of the wet gel varies accordingly with the speed of drying. On the outside the wet gel appears to pass also with a sharp boundary into dry gel which has a lower birefringence, 0.003. A shrinkage amounting to about 50 per cent—limited necessarily to one dimension—occurs in the drying out of wet gel. On further drying longitudinal, and less frequently, transverse cracks occur, leaving single pieces of approximate dimensions  $1.0 \times 0.5 \times 0.5$  mm., which can then be examined as are ordinary crystals by x-rays. This method of preparation of oriented surface layers of considerable thickness is one we have subsequently found to be of very general utility. By means of it we have prepared, for example, oriented specimens of nucleic acid and of the lipid containing antigen from *Brucella*.

Wet gel specimens for x-ray examination cannot be obtained in this way, as the material is softer than butter, and could not be removed from the cell without destroying its orientation. This difficulty can be overcome in two ways. A mass of wet gel can be reoriented by rolling between glass plates in a low temperature room to avoid loss of water. The oriented cylinders can then be transferred to the thin glass cells described below (p. 119). Alternatively, rewet gel can be used. A piece

of dry gel can be introduced into a narrow thin-walled tube and a suitable liquid—water, acid, or salt solution—can be introduced through a fine capillary. Except in extreme cases the gel swells without losing its orientation. Swelling can be limited by adding measured quantities of solvent, or equilibrium swelling reached by filling a section of the tube with the appropriate solution.

The “crystals” precipitated from solutions by acid (Bawden and Pirie, 1937a)<sup>2</sup> can be separated by centrifuging in the end of an L2 Chamberland filter candle (Bawden and Pirie, 1937a),<sup>3</sup> the buttery mass rolled and transferred into the special cells (described below) in the same way as for the completely wet gel, from which it is indistinguishable as will be subsequently shown (p. 129).

### *Optical Examination*

The optical studies on the virus were made with a Swift Dick polarising microscope. For examination of the solutions the simple cell described above was used, but where thicker preparations were required, free from the danger of evaporation, a cell consisting of two microscope slides separated from each other by a rubber sheet gasket and held together by brass clips was used (Text-fig. 3). Capillary tubes are also useful for certain purposes and their refractive effects were neutralised by immersing them between slide and cover-glass in a medium of suitable refractive index (Plate 2, Fig. 11).

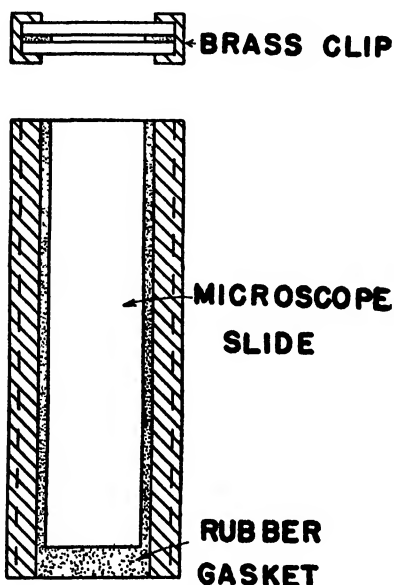
### *X-Ray Examination*

The standard x-ray apparatus used for the study of small crystals requires to be modified in two directions, both in order to measure low angle reflections and to enhance their intensity. To study adequately the large spacings which were observed, it was necessary either to use longer wave-length radiation or to examine the diffraction at smaller angles. The reason many of the phenomena here described were not discovered earlier was probably due to the choice of the first method. In deciding what radiation to use three factors must be borne in mind—the actual dispersion given by the radiation which at small angles is directly proportional to its wave-length, the absorption in all substances which is approximately proportional to the cube of the wave-length used, and the difficulty of producing the radiation which in turn depends on a number of factors but is certainly far greater for long wave-length radiation than for the standard copper  $K_{\alpha}$  radiation. The great advantage of using this radiation is that it is possible to work in air without vacuum cameras, and to use commercial x-ray tubes with large out-put. The necessary dispersion can be obtained most easily merely by increasing the specimen to film distance. It may, in the future, be necessary to work with long wave-length radiation, but for the moment all the preliminary work can most conveniently be done by changing the camera rather than the radiation. In the second place it was found that the reflection from many of the specimens was extremely feeble and means had to be used to increase the intensity of the scattered radiation, and the contrast achieved on the films. This was done by

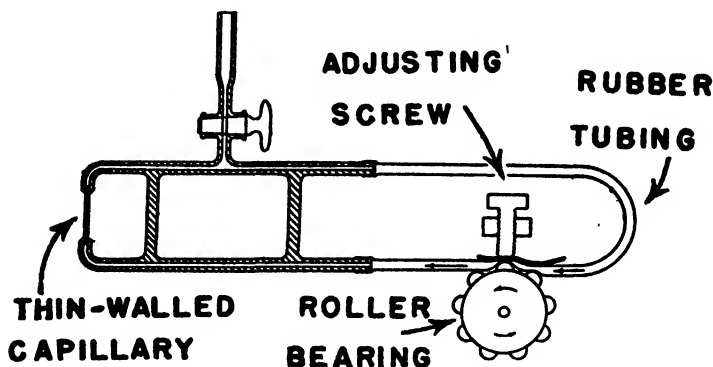
<sup>2</sup> Page 294.

<sup>3</sup> Page 300.

the adoption, where it was geometrically possible, of slits instead of the usual pin-holes, which enabled exposure times to be cut down considerably. Finally, particu-



TEXT-FIG. 3. Small cell for optical studies of tobacco mosaic virus proteins.



TEXT-FIG. 4. Peristaltic pump and glass circulatory system for x-ray examination of oriented top layer solution.

larly in the case of examination of liquids and wet gels, monochromatic radiation was used. The device introduced by one of us (Fankuchen, 1937) provided a source of monochromatic radiation, little if anything weaker than that provided by the direct radiation from the tube. Two types of cameras were accordingly used. A long camera for the intermolecular reflections was adapted from the normal Pye

camera (Bernal, 1928) by the addition of an arm 40 cm. in length, and the corresponding elongation of the slit system. The slits were made by adjusting lead jaws under the microscope to about 0.10 mm. in width. They were placed 15 cm. apart, and the scattering from the second slit was checked by a slit of 0.20 mm. near the crystal specimen. In this way it was possible to obtain a beam which, at a distance of 40 cm. was only 0.7 mm. wide, and therefore subtended an angle of  $5'$  at the crystal. It was possible to measure diffraction angles of  $4.5'$  corresponding to a spacing of 1200 Å. In some experiments the whole of the apparatus between the specimen and the plate was replaced by a vacuum camera, but without appreciable improvement. Besides this normal type of long distance camera, monochromatic slitless cameras were also constructed and used. The short cameras used for the intramolecular investigations were of the normal pin-hole type, but with specially small pin-holes giving a beam of divergence  $20'$ , thereby enabling plate distances up to 15 cm. to be used. The longest spacing measurable in this way is about 200 Å. Great care was taken to cut down the lead stop to a size allowing the maximum of pattern to be seen without any overlap of the central beam. It was found useful to use extremely thin lead back stops, leaving a faint trace of the central beam on the plate which could be used as a point of reference. With monochromatic radiation the lead stop could be replaced by one of a definite thickness of aluminium which allowed only a known proportion (1 per cent and 0.01 per cent) of the radiation to be transmitted and could therefore be used to estimate the absolute intensity of reflection.

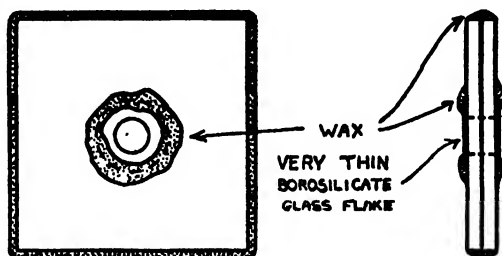
Of the specimens for x-ray examination only the dry gels could be mounted in air. For other preparations thin-walled tubes or special cells were used. It was found possible to make out of a special low absorption borosilicate glass, tubes of wall thickness of the order of 0.02 mm. These tubes were tested for x-ray absorption when empty and only those which absorbed less than a quarter of the incident characteristic copper  $K_{\alpha}$  radiation were used. There is no advantage in pushing the thinness of the capillaries very much further, because the water with which the specimens are enclosed produces more absorption than the glass.

The specimen was usually secured against change in the capillary by sealing the ends in a small flame. It was of great importance to have absolute sealing to avoid any change in the specimen during the long exposures which often proved necessary.

These capillaries were used mainly for specimens of wet gel precipitated crystals, and for bottom layer solutions of various strengths. For top layer solution, in order to produce orientation, it was necessary that the liquid should be flowing. A small apparatus was therefore prepared, consisting of a stout capillary tube system, on to which was sealed a piece of thin capillary, the liquid being maintained in motion by means of a peristaltic rubber tube motor (Text-fig. 4). In this way both top solution and precipitated crystals were examined in the oriented state.

The disadvantage of capillary tube methods is that specimens which are easily deformable cannot conveniently be introduced into them. For x-ray examinations of such specimens a small brass cell was used. This was prepared as follows. Two brass plates of dimensions  $7 \times 7 \times 1$  mm. were accurately ground together and a hole of 2 mm. diameter drilled through them. To the ends of this hole were sealed sheets of borosilicate glass blown to bubble thinness. The brass plates were then sealed together with sealing wax. Just before using, the seal was broken with a sharp

pointed knife (Text-fig. 5). Into this cell the specimen was introduced and the plates were then sealed by closing the fine crack in the sealing wax with a heated knife blade. All these operations were carried out in a low temperature room to avoid evaporation. Such cells showed no appreciable loss of water over periods of many days. It might be thought that the scattering of the glass, which though small in volume is of the same order as that of the scattering material to be studied would affect the photographs. This, however, was not found to be so. A certain amount of scattering did occur, but it was in a range equivalent to  $4\text{--}2\text{ \AA}$ , and therefore at angles which were much too high to interfere with any of the significant scattering from the specimens. The same was largely true of the water scattering. It is an interesting but not unexpected fact that prolonged exposures show no appreciable scattering for the water or glass at very low angles. The slight scattering that did occur at low angles was subsequently shown to be largely due to the air in the apparatus, as most of it could be removed by using a vacuum camera.



TEXT-FIG. 5. Small cell for x-ray study of wet gels.

## II. The Modes of Aggregation of Virus Particles

This section is conveniently divisible into two main parts, first a description of the x-ray and physicochemical observations on modes of aggregation of the virus, and the second a discussion of these observations in the light of a general theory of equilibrium between colloid particles.

### A. The Observations

All the viruses examined, except bushy stunt, have one common characteristic: their capacity for forming doubly refracting aggregates. In the case of the dilute top layer solutions this is only produced by flow orientation. In bottom layer solutions and gels, the orientation is spontaneous, any small region of the material behaving as a uniaxial positive crystal. Normally, these regions have different orientations, but by flowing, rolling, and the action of neighbouring surfaces, for example in narrow tubes or on air-water interfaces, large portions of the material can acquire the same orientation. The orientation of the wet gel is retained on drying. The needle-shaped "crystals"

precipitated by acid or ammonium sulfate, the tactoids of bottom layer solution occurring in top layer solutions (see p. 128), and the hexagonal crystals occurring in infected plants also show positive birefringence.

The simplest explanation of this property is that it is due to the existence in all the phases of markedly anisotropic particles, which have a tendency to set themselves parallel. X-ray examination confirms and gives precision to this hypothesis.

When oriented preparations of dry and wet gels and solutions of strengths down to 8 per cent are examined in the low angle x-ray camera described above, a pattern of reflections is observed characteristic only of the concentration of the specimen (Plate 3, Figs. 15 and 16). This is called, for reasons that will be discussed below, the intermolecular pattern. All these reflections occur on the equatorial line in the photograph, that is they correspond to planes parallel to the axis of orientation. The most careful search was made for other intermolecular reflections, particularly from planes perpendicular to the orientation axis, but it failed to reveal any such reflections. The most complete intermolecular pattern is given by dry gels. There are four strong reflections, but three other weaker ones have been observed, particularly well developed in the cucumber virus (Plate 3, Fig. 18). (The potato virus X gives no observable intermolecular pattern, see below p. 159.)

These reflections correspond to the first four planes of a two dimensional hexagonal packing of side  $152 \text{ \AA}$  ( $146 \text{ \AA}$  for the cucumber virus). The agreement of the spacings with those calculated for a hexagonal lattice is perfect (see Table I); any variation from it would lead to blurring of the outer lines, which does not occur. The lines are in fact remarkably sharp, indicating that the areas of regular packing are large compared to the size of the particles. Actually, as will be shown later, the effective area may extend to that of the whole specimen. The patterns for the wet gels, for the precipitated "crystals," and for the solutions, also correspond to a hexagonal packing, with spacings increasing with the dilution; but here it is rarely possible to distinguish more than three lines in the first two cases and two in the last, the ratios of their spacings still indicate, however, the hexagonal nature of the packing. The full data are given in Table I.

The existence of hexagonal packing and the absence of any intermolecular reflections from planes not parallel to the axis of birefringence point to the existence of particles of identical cross-section, not less than  $150 \text{ \AA}$  in diameter which have a tendency to pack in parallel bundles but give little information as to the lengths beyond setting a minimum value of about  $1500 \text{ \AA}$ . We will here attempt to relate the observed properties of the virus at different concentrations to this hypothesis and in particular to discuss the transition between the ordered and disordered phases. It will be convenient to discuss first of all the bottom layer and top layer solutions and from there to pass to a discussion of



the gels, bearing in mind, however, that the bottom layer solutions, the wet and dry gels, all form part of the same ordered phase.

### *Bottom Layer Solutions*

As has already been mentioned, solutions of the virus of concentrations between 1.8 per cent and 34 per cent if purified and of between 8 per cent and 34 per cent if less pure form spontaneously birefringent liquids whose properties change continuously with the concentration. The solution at extreme dilution appears clear except for a characteristic sheen, and mobile, possessing a low but anomalous viscosity. While the more concentrated solutions are extremely viscous, they can be made to flow through capillaries, but they will not flow under their own weight, possessing a certain degree of elasticity which might lead to their being considered as gels (see p. 131). Such centrifuged preparations from the solutions are not distinguishable by x-ray methods from dry gels swelled at high pH or in dilute salt solution.

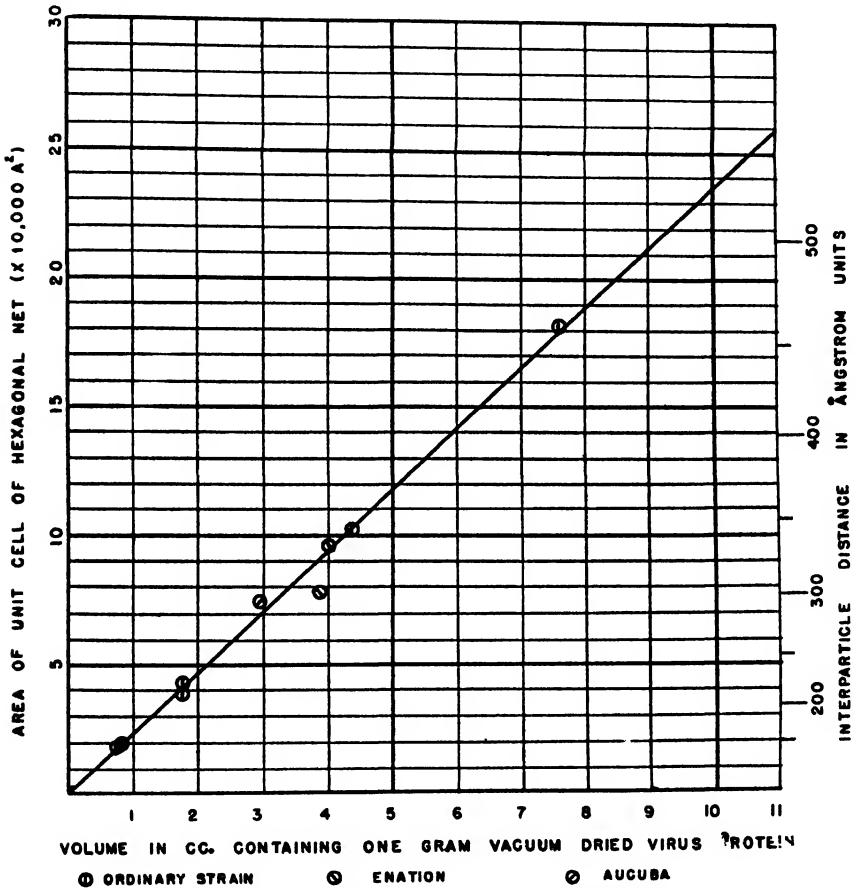
Examination of the liquid in bulk shows that it is divided into birefringent regions, which, in the still liquid, have no discernible shape or relation to each other. The size of these regions depends on the concentration of the virus. It is largest in the most dilute and smallest in the most concentrated solutions. Examined in thin layers, the liquid has the appearance shown in Plate 1, Fig. 4; the areas are seen not to be sharply defined regions, but represent a continuous flux of direction of birefringence in layers of 0.5 mm. or so, showing orders up to the 3rd, but there are no really black regions, indicating that parallelism to the glass sides does not extend through the distance between them but instead that there are twisted formations showing optical activity as can easily be seen by rotating one of the nicols.

In extremely thin layers, regions of approximately constant orientation appeared, the direction of maximum birefringence varying over the field in a sinuous manner. There is a marked tendency for orientation to take place parallel to the glass and optically active layers can be artificially produced by rotating the cover-slide over the glass head as in the case of nematic liquid crystals (Friedel and Friedel, 1931).

In flow the maximum refractive index sets itself along the flow lines, but when the flow ceases this arrangement is maintained indefinitely. Plate 1, Fig. 2 shows the effect of flow very clearly. The liquid is flowing through a narrow aperture between the glass tube spacing rod and a layer of wet gel. On the side from which the flow comes the orientation lies parallel to a set of stream lines converging on the orifice, while on the other side flow consists of loops alternatively to the right and left of the orifice.

In thin tubes there is in general after a little time complete orientation with the direction of maximum refractive index along the axis of the tube. The extinction direction is as sharply defined as in a true crystal, showing a definite

mean orientation of the particles. Pin-hole x-ray photographs taken at film distance of 12 cm. and 15 cm. (Plate 4, Fig. 24, and p. 136) show that most of the particles have this mean orientation; the angular scatter of the particle axis is not more than  $1.5^\circ$  and is probably much less as this measurement makes no allowance for the lack of resolving power of the particle reflections.



TEXT-FIG. 6. Relation between virus concentration and interparticle distance.

### *Regularity of Structure*

Photographs taken with the special camera at 20 cm. or 40 cm. plate distance show two large spacing lines, which are in well oriented specimens, very sharp (Plate 3, Fig. 17) as well as a diffuse line at  $93 \text{ \AA}$  which does not vary with the concentration and is consequently produced by the particle and not by the arrangement of particles (see p. 153). As has already been mentioned, the sharp lines correspond to the two first reflections (1010) and (1120) of hexagonal

packing of variable interparticle distance. Measurements of this distance brought to light an extremely simple regularity. The distance between the particles is inversely proportional to the square root of the concentration by volume and is expressed by the equation  $R = 1650/\sqrt{N}$  where  $R$  is the interparticle distance in Å units and  $N$  the number of grams of vacuum dried virus in 100 cc. of specimen. The immediate consequence of the existence of this relation is that the *particles distribute themselves in a hexagonal array so as to fill the available space as uniformly as possible.*

This is shown in Table II and in Text-fig. 6 by plotting the area of a unit cell of the hexagonal arrangement against the volume of solution associated with unit weight of dried virus. For percentage concentrations ranging from 100 per cent for the dry gel down to 13 per cent the points lie, within the experimental error, on a straight line through the origin.

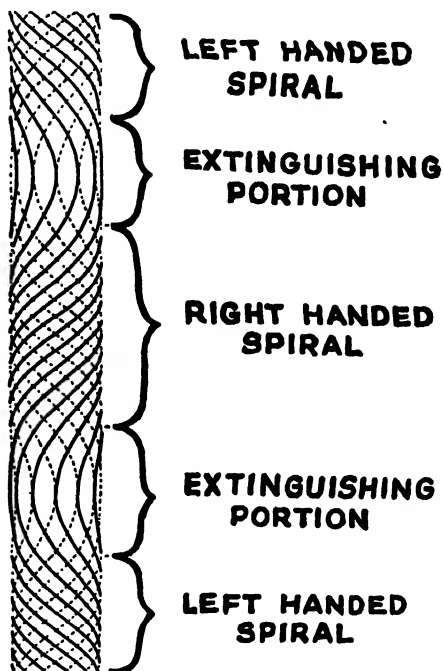
A further implication of the relation between spacing and concentration is that on dilution, the particles separate measurably only in the direction at right angles to their length. If they also separated to any considerable extent in the direction of their length the lateral separation would not increase at the rate observed. This suggests that the particles may form long chains in the solution and even that there may be some form of attachment between their ends. Such attachments have been observed by Kausche *et al.* (1939). The observation of a regular lattice arrangement of colloidal particles in solution and at distances up to 500 Å—more than three times the diameter of the particles—may prove to be of cardinal importance for the theory of such solutions. The discussion of its physical basis will be left till later.

### *Reversed Spirals*

Before leaving the description of the bottom layer solutions, it is worth mentioning another typical structure which they exhibit, that of reversed spirals. When the liquid is made to flow rapidly through a fine tube, or alternatively the tube is set vibrating by bending one end and releasing it, it appears between crossed nicols to show striations which divide it, when the nicols are parallel to the tube, into light and dark regions (Plate 2, Figs. 7 and 8). This is a phenomena first observed by Van Iterson (1934) in cadmium glycerophosphate. On turning both nicols it can be seen that the light regions are never extinguished, but that they can be made to do so if one nicol is turned, showing that the light regions are alternatively dextro- and laevorotary, while the dark regions separating them behave like optically inactive crystals oriented parallel to the tube. The explanation of these optical effects is clearly that here the long axes of the particles set themselves spirally in the tube, these spirals being alternately right and left handed, separated only by regions where the particles lie parallel to the tube axis (Text-fig. 7). The reverse spiral arrangement for liquid flow is a further confirmation of the

existence of very long particles. Eirich and Simha (1937) have shown, experimentally as well as theoretically, that if parallel oriented particles experience resistance on flowing through a tube a periodical change of orientation is the most stable arrangement.

Similar phenomena are shown in the drying gel, in capillary tubes or on plates, (Plate 2, Fig. 10), but in this case a zigzag instead of a spiral arrangement is produced. These phenomena are of interest because of their close resem-



TEXT-FIG. 7. Directions of molecular axes in reversed spiral arrangement on surface of circular cylinder.

blance to many natural structures, for examples, to the striations of muscle (Bernal 1937), and this suggests very strongly that all are due to the same cause—the presence of long particles with a tendency to parallel orientation.

#### *Top Layer Solutions*

The top layer solutions are slightly opalescent, markedly more so than the bottom layer though they contain less solid material. This indicates at once their greater optical inhomogeneity due to their lack of regular arrangement. Lauffer (1938a) has studied polarisation of the Tyndall effect on these solutions and confirms the presence of long particles. The birefringence of the particles is, in his opinion, rod birefringence and not intrinsic. The proofs adduced are,

however, not altogether convincing as the influence of the medium on the particles cannot be excluded. Mere loss of birefringence might have been produced by disaggregation and no increase of birefringence after passing a minimum was demonstrated. Nevertheless some rod birefringence must be present to account for the observed diminution of birefringence between wet and dry gels (see p. 129). The chief properties top layer solutions exhibit are flow anisotropy and anomalous viscosity. Both of these and the relation between them have been studied by Robinson (1938; 1939 *a* and *b*) and his conclusions will only be mentioned here in so far as they throw light on the shape of the particles.

### *Flow Anisotropy and Viscosity*

In any flow having a velocity gradient, the particles are oriented in relation to the stream lines, but do not, except for high speeds or very long particles, lie in them. For the most part they turn end for end in an irregular manner about axes in the planes of flow perpendicular to the stream lines, spending most of the time in a position near to but not precisely at that of the stream lines, as brownian motion tends to produce an equilibrium position making a definite angle with them. This is the complement of the so-called angle of isocline, observed in flow birefringent solutions. This process gives rise to an anomalous viscosity, that is, to a frictional loss of energy increasing less rapidly than the rate of shear. Robinson has shown that these phenomena are affected by temperature in the way this theory would indicate. Rise of temperature brought about diminished flow birefringence, increased deviation of isocline, and increased anomalous viscosity. From his measurements it was possible to obtain an estimate of the ratio of length to thickness of the tobacco mosaic virus particles in the preparation he used as 88 to 1, a measurement which will be discussed later.

The solutions studied by Robinson were extremely dilute—0.02 per cent—and the particles moved completely independently. In more concentrated solutions approaching the limiting concentration of the phase 1.8 per cent to 4 per cent, there is evidence of interaction between the particles in solution, elastic viscosity begins to appear, and the flow birefringence does not disappear immediately on stopping the flow but persists for a longer or shorter time according to the degree of purification of the virus.

### *Effects of Electric Currents*

That there is an appreciable time of relaxation in strong top layer solutions is also shown by their behaviour in electric fields. A small cell with platinum electrodes about 1 mm. apart was used to study this effect and was observed between crossed nicols. The effect of passing a direct current across the cell was an immediate luminosity around the anode. This luminosity was quite

clearly due to the cataphoretic concentration of the virus, the particles of which carry a negative charge, and the consequent formation of spontaneously birefringent bottom layer. With low voltage—less than 1 volt—the effect was reversible. Reversing the current caused the disappearance of luminosity at one electrode, and its appearance at the other, the two being bridged by fine streamers. With higher voltages, irreversible changes took place involving the permanent formation of birefringent gel.

With alternating current, as long as the voltage was low—less than 5 volts—no such appearance of the bottom layer was observable. Instead, the switching on of the current immediately gave rise to a faint luminosity between the electrodes, this luminosity being clearly due to the orientation of the top layer itself (Plate 1, Fig. 6). The luminosity appeared the moment the current was turned on, but did not at once disappear when the current was broken, remaining for a period, estimated to be between 1/10th and 1/5th of a second.

These experiments indicate that the anisotropic virus particles are easily oriented, but that this orientation is brought about, as in many other liquid crystals forms, more by the cataphoretic currents produced by the electric field than by the direct orientation effect of the field in part of it, as is also the case with many liquid crystals (Fredericksz and Zolina, 1933). This is confirmed by our failure to observe any orientation in a magnetic field of 5000 gauss. Lauffer (1939) has made extended observations on electric orientation of tobacco mosaic virus. He ascribes it to dipole effects but his observations are not inconsistent with the explanation given above.

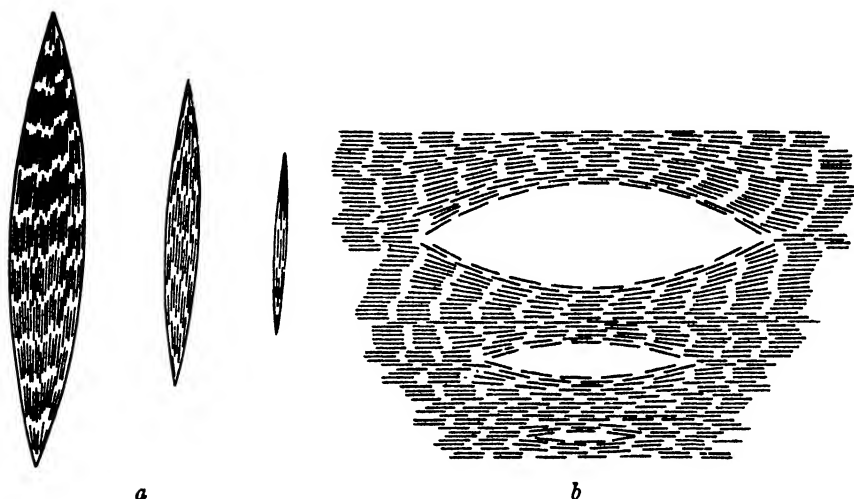
### *X-Ray Examination*

The x-ray examination of the top layer solution carried out in the continuous flow apparatus, already described, confirmed the picture of the almost complete orientation. No intermolecular pattern was found, but this was not surprising as the calculated mean interparticle distance should have been of the order of 1500 Å, nor would regular interparticle distances be expected in any case. On the other hand, the most intense reflection of the intramolecular pattern (0006) (see p. 148) appeared in the form of arcs which were sharp and of small angular width, indicating at the same time the parallel orientation of the particles and their identity with those of the ordered phase.

### *Equilibria between Top and Bottom Layer Solutions: The Tactoids*

In the initial separation of top and bottom layers from a virus solution and in any subsequent shaking together of the layers, characteristic spindle-shaped bodies are observed (Plate 1, Figs. 3 and 4). Similar bodies have been observed in other colloidal solutions and named tactoids by Zocher (1925, 1929) (see also p. 131). The tactoids are of two kinds, which we have called positive and negative. Positive tactoids contain bottom layer in top layer

solution, negative top in bottom. In Plate 1, Figs. 3 and 4, both are shown. Both kinds of tactoids have when undistorted a roughly toroidal surface; in outline they appear as two intersecting circles with slightly cusped ends. Rough measurements show that the minimum radius of curvature of all tactoids in a given preparation diminishes more slowly than their absolute size so that large tactoids approach a spherical shape and small ones are needle-shaped. The radius varies, however, enormously with the age and ionic constitution of the specimen. For fresh bottom layer of 4.5 per cent, salt free and pH 7, it gives the smallest values which are of the order of 0.06



TEXT-FIG. 8. (a) Orientation of particles in positive tactoids of different sizes (diagrammatic). (b) Orientation of particles around negative tactoids of different sizes (diagrammatic).

mm. The internal structure of the positive tactoids is revealed by their appearance between crossed nicols, the slow extinction position, and consequently the axes of the particles following the meridian lines of the spindles (Text-fig. 8a). In the negative tactoids the outline depends on the arrangement in the surrounding bottom layer solution (Text-fig. 8b). The tactoids, particularly the larger ones, are easily distorted, but recover their shape; they can also fuse to give larger tactoids. This suggests that they represent an equilibrium form between the conditions of surface energy tending to produce a spherical shape and those of orientation potentials tending to keep all the particles approximately parallel. A roughly quantitative theory of tactoid shape can be built up on this basis, but it is best considered after the discussion of interparticle equilibria (see p. 140).

### *The Gels*

The forms taken by the more concentrated virus preparations prepared by drying and precipitation were originally thought to consist of two different phases, dry and wet gel, showing constant characteristics. This opinion was held both for experimental and theoretical reasons (Bawden *et al.*, 1936). On drying sharp boundaries seemed to separate the drier outer portion from the inner portion and this in turn from the solution. Further, the wet material and the "crystals" prepared by precipitation were alike in their physical properties, having a dry virus content of between 45 per cent and 55 per cent, and giving the same intermolecular x-ray pattern, showing an interparticle distance of 200–210 Å. There seemed therefore to be a definite phase which it was possible to account for only on the assumption of the swelling of the particles, as at that time no other forces of known character could be postulated holding dry particles in equilibrium at such a distance. Subsequent work, however, showed this view to be incorrect, and it has been replaced by a much simpler one, namely that all concentrations of the virus from 100 per cent–2 per cent form one ordered phase, consisting of a regular hexagonal packed assembly of parallel particles their distance apart varying from 150–1000 Å. The particles retain substantially the same internal structure in all states. The nature of the forces between them will be discussed later, but it is first necessary to summarise the experimental evidence.

### *Dry Gel*

The study of dry gel has been more thorough than that of any of the other phases on account of its ease of manipulation. The intermolecular pattern is particularly sharp and shows the greatest number of lines, indicating extreme regularity of hexagonal packing of the particles with an interparticle distance of 152 Å. This is a surprisingly constant number for air-dried gels of all tobacco mosaic strains, formed in many different ways. Drying over  $P_2O_5$  in a vacuum reduces this distance to 150 Å. There appears to be a sharp distinction in physical properties between dry and wet gel. The material is hard and horny, its birefringence is only half as great, 0.003. This is due to an increase in the transverse refractive index from 1.484 to 1.532 from wet to dry gel due to close apposition of the rod-shaped particles while the longitudinal index rises only from 1.490 to 1.536.

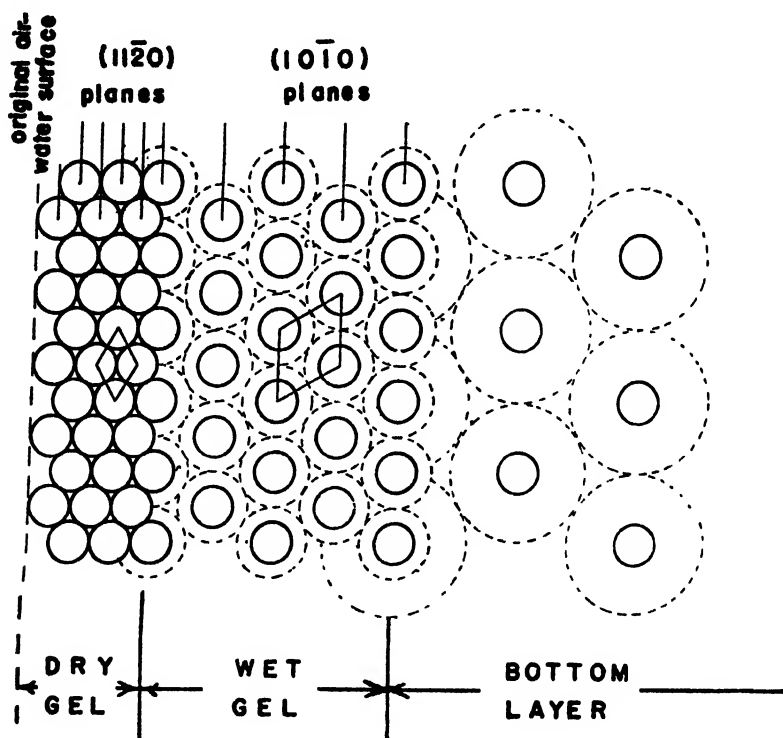
### *Single Crystal Character*

Apart from its intramolecular structure, its chief interest is in its relation to wet gel. For this the cardinal observation (see p. 147) was that pieces of dry gel behaved as single crystals in two dimensions. An intermolecular photograph with the x-rays parallel to the axis of orientation showed a distinct hexagonal pattern (Plate 4, Fig. 22). It is significant that the corresponding



intramolecular pattern (Plate 4, Fig. 23) showed only continuous rings, indicating that at least in the dry gel the orientation did not extend to the inner structure of the particles.

Now, in the mode of preparation of the gel by drying, two factors may affect the transverse orientation of the particles, that of the air-water and glass-water interfaces which are at right angles (Text-fig. 2). Of these, the



TEXT-FIG. 9. Section across particles in successive layers of drying film to illustrate orientation of "single" crystal of dry gel.

effect of the first is likely to be predominating. Now it is at first sight strange that the most densely packed plane of the hexagonal packing (1100) is found parallel not to the air-water but to the glass-water interface, while the former is occupied by the less densely packed plane (1120). The rigid dry gel, however, cannot have oriented itself in the dry state; its orientation must have been acquired in the wet state and simply fixed by drying. Now the shrinkage of about 50 per cent of the wet gel on drying takes place only in the direction perpendicular to the air-water interface. It is natural, therefore, to assume that this brought about the interchange of the hexagonal packing directions

(Text-fig. 9), and that in wet gel the (1100) plane is parallel to the air-water interface. Thus in the wet gel the particles would be closely packed in this surface, a very reasonable arrangement. The rearrangement could not be perfect owing to the lack of fit of the interparticle dimensions and this may give rise to the cracks that are observed in the dry gel.

### *Non-Equilibrium Gels*

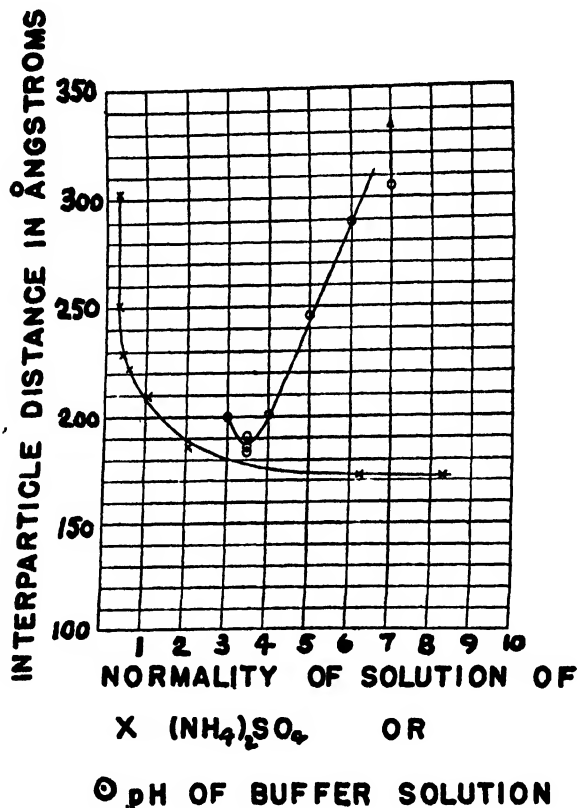
Dry gels formed from solutions of the virus prepared at pH 7 will if placed in salt-free water at the same pH swell indefinitely and dissolve to reform reversibly the oriented phase from which they were prepared. X-ray studies show that here the distance between the particles is always derivable from the concentration (see p. 124, and Text-fig. 6), and this holds as well for the gels as for the more dilute solutions. Additions of small amounts of water to dry gels result in interparticle distances intermediate between the values of 150 Å and 210 Å previously supposed to be characteristic for wet and dry gel and thus makes it very unlikely that they are distinct phases (but see p. 140). Similarly, values between 210 Å and 290 Å, the value for the most concentrated solution, have also been found. This establishes the complete continuity of the non-equilibrium gels and of the bottom layer solutions previously described as one continuous oriented phase of the virus. The state of the particles in this phase is called one of non-equilibrium as they tend to move further apart if given more room as do molecules in solution or particles in a gas, but they differ from them in that they are arranged in a regular lattice structure. They behave in fact as crystals with a long range elasticity.

### *Equilibrium Gels*

Virus solutions near the isoelectric point at pH 3.4 in salt solution are precipitated in the form of fibres or acicular "crystals" containing a variable amount of water. The same phenomenon occurs with a variety of other agents, alcohol, acetone, or merely by ageing. X-ray examination shows that these precipitates are identical with those formed by allowing the dry gel to swell in the same liquids. This indicates that in these cases an equilibrium is attained determining the water content of the gels which may consequently be called equilibrium gels. The study of these gels by x-rays shows that here the distance between the particles is independent of the amount of medium present and depends only on its character. This dependence has been studied in a preliminary manner in two series, that of pH and of ammonium sulfate solution. The results are shown in Table III and Text-fig. 10.

Pieces of dry gel were swelled in a number of solutions of citrate-phosphate buffer of pH varying from 2.5–7. There was a definite minimum at pH 3.4 with an interparticle distance of 185 Å, while at the higher pH the distance increased to 300 Å, greater than that of some of the bottom layer

solutions previously examined. The salt solutions gave similar results except that here there seemed a definite saturation effect, solutions stronger than 6 N giving almost the same minimum distance, 173 Å. The minimum would seem therefore to have some special importance being the least distance of approach of the particles in an aqueous medium. The existence of inter-

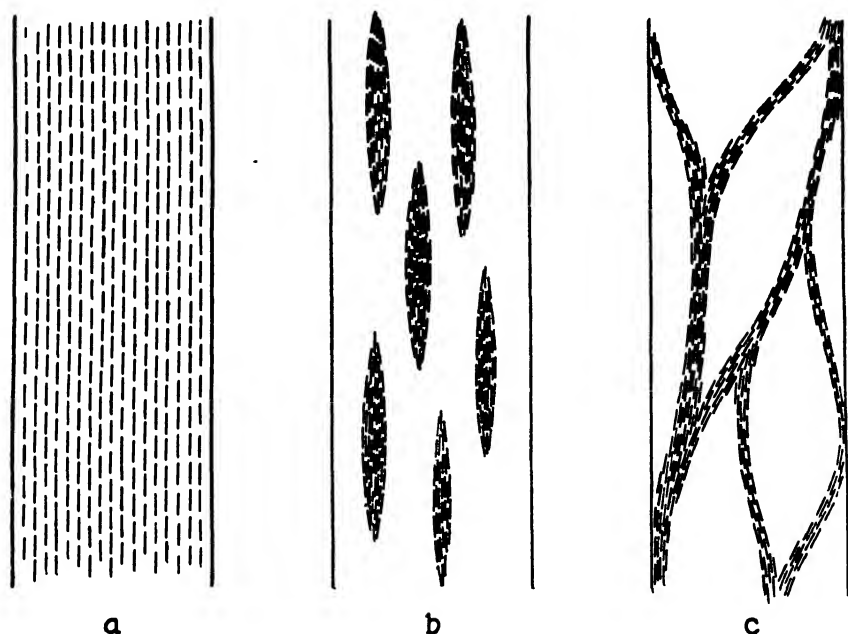


TEXT-FIG. 10. Relation of interparticle spacing and concentration of salt solution and pH.

particle distances of between 210 Å and 230 Å for many concentrations of salt and acid explains how it was possible for so long to consider the gel of this composition, *ca.* 50 per cent dry weight, as a definite phase—wet gel. The state of the virus here is probably physically analogous to that of protein crystals which usually contain about the same proportion of water; it will be discussed in a subsequent section.

\* It may be pointed out here that the x-ray method gives an exact way of estimating the intrinsic water content of a gel, namely that associated with the

gel particles. The apparent water content may be much greater. Thus Bawden and Pirie (1937*a*) observed that the first effect of decreasing the pH of a virus solution was the formation of a gel which might contain 95 per cent or more water. On shaking these gels separated out as "crystals" which contained 50 per cent water. Our observations, particularly those in which acid solutions were allowed to diffuse into already oriented virus solutions,



TEXT-FIG. 11. Rheopectic effects in acid-precipitated virus solution (diagrammatic). (a) Oriented 4 per cent virus solution in capillary tube: free flowing. (b) Effect of slow precipitation with acid. Formation of independent tactoids: free flowing. (c) Effect of agitation: fusion of tactoids: gel formation.

showed that in all cases submicroscopic fibres of this composition were produced and these being already parallel did not form a gel and flowed readily (Text-fig. 11) though more dilute disoriented solutions did so and showed marked rigidity. It seems clear therefore that in gels of these substances and by inference in those of many others containing long particles of colloidal dimensions we should distinguish two mechanisms of water retention. Such gels contain both intrinsic water lying between the most closely apposed particles and depending only on the nature of the solvent, and trapped water depending on the concentration of gel substance and the conditions of gel formations and ageing.

The observations on the conditions of equilibrium of virus gels are still in

their earliest stage. The effect of other conditions, temperature, non-electrolytes, etc., have still to be studied. Their interest is primarily that of colloid physics; as far as the nature of the virus is concerned they have sufficed to show that all its properties in an aqueous medium are explicable in terms of long particles reacting with each other through the medium in such a way as to keep them parallel and equidistant at all concentrations above a critical one depending on the nature of the medium.

### *The Crystals in Infected Plants*

All the forms of virus preparations hitherto described seem composed of distinct particles of a length far greater than their diameter. This length, however, seems to be a variable quantity and is very probably an artefact (see p. 135). On the state of the virus in the plant we have only two observations to go on; the weakness of flow double refraction (see p. 135) and the existence of crystals in some infected plants. These crystals, which have been studied by Bawden and Sheffield (1939) and Beale (1937) probably do not represent the virus in a pure form. Their solubility is too small, but they may well be compounds of the virus with cell proteins (Bawden and Pirie, 1937 *a*).<sup>4</sup> That they are related to the virus is shown by their association with infection, by their hexagonal habit and positive birefringence, which correspond to the particle arrangement and optical character of the gels, and by their breaking up into bundles of needle-shaped acid precipitate "crystals" on acidifying the sap. More recently, Kausche (1939) has demonstrated the reverse process by producing minute hexagonal crystals from a mixture of infected sap and precipitate "crystal." If we grant this relationship, the hexagonal crystals throw much light on the state of the virus in the plant. Unfortunately, they have proved too small and unstable to be submitted to x-ray examination, but from optical observation certain conclusions can be drawn. The crystals are bounded by hexagonal prisms and basal pinacoids. The existence of the last shows that here we must have a full three dimensional regularity and therefore particles of identical length.

In a few of the many specimens examined it was thought that acute angle pyramids could be observed, but the angle could not be measured so that the axial ratio of the crystals remains undetermined. The habit of the crystals with a width-thickness ratio of approximately four to one indicates according to Fedorof's rule that the length-thickness ratio of the molecules which make it up must be of somewhat the same order, giving a particle length of the order of 1000 Å.

The ready transition to the needle form shows that the particles are already aligned in the crystal and the fact that little swelling is observed shows that their distance apart is not markedly different from that of the isoelectric gel,

namely 185 Å. The observations are necessarily very qualitative, but they point in the same direction as those arrived at in other ways. If sufficiently powerful x-ray apparatus were available the crystals would undoubtedly furnish far more precise information on the nature of the virus than is furnished by the less regular gels and liquid crystals. It is convenient at this point to present evidence as to the length of the particles, since it plays a rôle of cardinal importance in the discussion of interparticle equilibrium.

### *The Length of the Particles*

X-ray evidence as to the length of the particles is all negative, that is to say no reflections attributable to regularities greater than the intramolecular regularities have been observed. The sharpness of these, however, indicates that the particle length must be considerably larger than its width, certainly over 1000 Å. Various physical methods (ultracentrifuge, viscosity, birefringence of flow, diffusion) (Eriksson-Quensel and Svedberg, 1936, Bechhold and Schlesinger, 1933, Wyckoff, 1937*a, b*, Wyckoff *et al.*, 1937, Stanley, 1938, Lauffer, 1938*b, c*, Takahashi and Rawlins, 1932, 1933*a, b*, 1937, Lauffer and Stanley, 1938, 1939, Bawden and Pirie, 1937*a, b*, Bawden *et al.*, 1936, Hills and Vinson, 1938, Staudinger, 1932, Neurath, 1938, Neurath and Saum, 1938, Mehl, 1938) provide other bases for estimating the length. All agree in indicating a considerable length for the particles, but although none of the methods was particularly exact, it was clear that the discrepancies between them exceeded the probable experimental error. The conclusion which follows from the remarks made above is that the particles might be of indefinite length depending on the mode of preparation, that they are probably shortest in the living plant, and are increased in length by the methods of purification employed. Fortunately, it is now not necessary to consider this evidence in detail as the study of the virus by means of the electron microscope by Kausche, Pfankuch, and Ruska (1939), and Marton (1941) enables the length to be measured directly. The particles as seen in the electron microscope are rods of different lengths, but they are all multiples of a length approximately 1500 Å, showing that the particles are homogeneous in respect of length, as indicated by the existence of true crystals in the plant. The preparations studied by various workers were all probably aggregated to different degrees. However, recent work of Loring, Lauffer, and Stanley (1938) suggests that preparations may be obtained with little if any aggregation. For instance that studied by Robinson must have consisted of particles containing from 8–10 Kausche units. The length of 1500 Å for the particles is sufficient to contain 22 repeat units as determined from the intramolecular observations (see p. 153), but 24 is not excluded. It is also very likely that the virus as observed by Kausche *et al.* (1939) was already somewhat aggregated and that groups of 12 or less repeat units may occur in the plant, though there is no positive evidence of this.

### *B. Interpretation*

#### *The Nature of the Interparticle Equilibria*

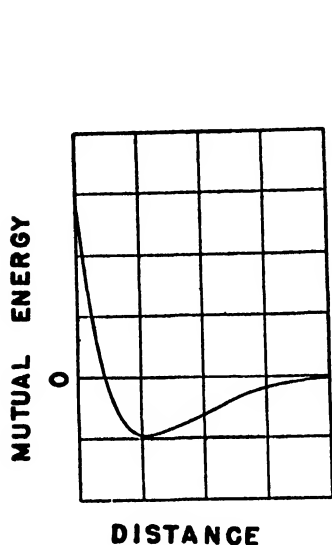
From the point of view of colloid science the most interesting observation to which the x-ray study of virus preparations has led is the regularity of packing of the long virus particles, not only in the concentrated gels but also in solution. That such good order should be attainable in the dry state was unforeseen although not unprecedented, but in the case of the solutions where the particles are separated by water molecules, a distance much greater than their diameter, 150 Å, crystalline regularity was certainly not to be expected on prevailing views. At most it would have been assumed that a roughly even distribution of the particles would be maintained but that anything more regular would have been prevented by thermal motion. Observation shows, however, that the solutions down to a concentration of 20 per cent show as perfect reflections as a crystal. More dilute solutions, down to 13 per cent, have not been so carefully examined and indeed show slightly less sharp maxima, but this is probably due to inhomogeneity rather than irregularity, for similar broadenings were previously observed on stronger solutions but disappeared when well orientated and homogenized specimens were used. As the continuity of physical properties is apparently unbroken down to the critical concentration of 1.8 per cent, it is probable that these are also regular, though, owing to difficulties of observation of such small angles and the weakness of the reflections, this has not yet been proved.

Not only are the particles equidistant, they are arranged in almost exact parallelism. The intramolecular photographs of 36 per cent (Plate 4, Figs. 24 and 25) solution show that the mean deviation from parallelism does not exceed 45', and that it is probably far less. The same is shown in more dilute solutions by their sharp extinction when observed in polarised light. On the surface of the tactoids the particles are constrained by strong surface forces, but even here the angle of deviation, as measured by the minimum radius of curvature in the longitudinal direction, is still small. The observed radius of curvature of 0.06 mm. gives for a particle of length 1500 Å a maximum particle to particle deviation in this direction of only 8'.

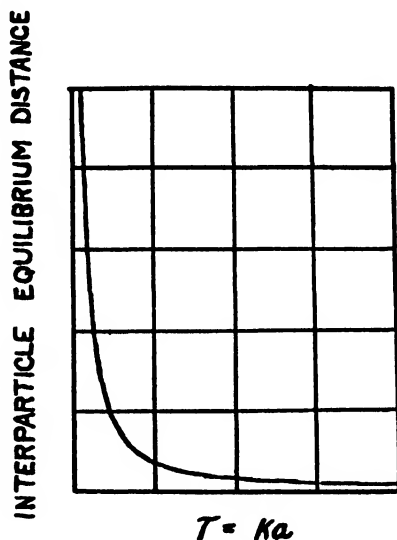
Such equidistance and parallelism can only be maintained by definite forces acting between the particles. For concentrations greater than the critical concentration of 1.8 per cent in the absence of acid or salts, these forces must have a net repulsive character, as is known by the formation of non-equilibrium gels, but at this concentration attractive forces must balance them or no division into layers would be possible. The seat of the forces cannot be in the particles alone. London-Van der Waals forces are ineffective at such distances and in any case give attractive forces. The most probable explanation lies in the Debye ionic atmospheres around the particles which at  $\text{pH} > 3.4$  carry a net negative charge.

*The Theory of Particle Interaction*

The theory of the interaction of particles in an ionic medium has recently been studied by several authors, notably by Langmuir and Levine. Langmuir's theory (Langmuir, 1938) has so far been worked out only in the case of infinite plane particles which would correspond in practice to those of hydroxide sols or clay suspensions. Levine's treatment, which deals with spherical (Levine, 1939*a* and *b*) and later with cylindrical (Levine, 1939*c*)



TEXT-FIG. 12



TEXT-FIG. 13

TEXT-FIG. 12. Mutual energy of two spherical particles in an ionic medium as a function of distance (from Levine, S., *Proc. Roy. Soc. London, Series A*, 1939, 170, 152).

TEXT-FIG. 13. Interparticle equilibrium distance as a function of ion concentration (from Levine, S., *Proc. Roy. Soc. London, Series A*, 1939, 170, 152).

particles, is more directly applicable for our case. Physically the problem is very similar to that of the interaction of metal atoms with their positive nuclei and cloud of negative electrons, but on a scale comparable to that of the particles themselves. The simplest case treated by Levine is that of spherical particles. He is able to show that for two charged spherical particles there is always a potential energy distance curve of the type shown in Text-fig. 12, namely one which exhibits attraction at large distances and repulsion at smaller ones, and provides an equilibrium position with a minimum of potential energy. The position and depth of the equilibrium depends only on the product

$$\tau = Ka = \frac{a\sqrt{\gamma}}{3.04 \times 10^{-8}}$$

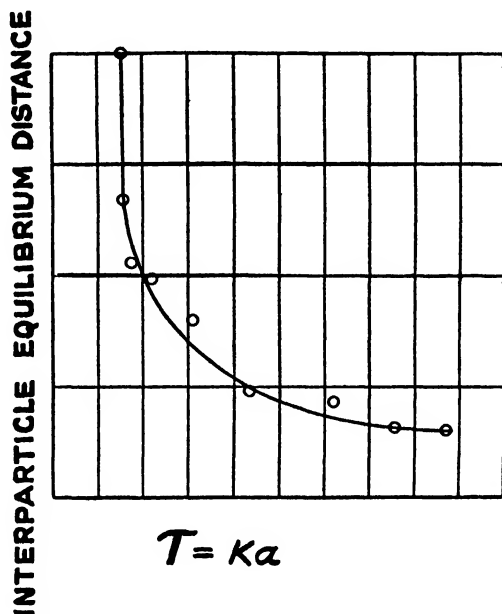


where  $a$  is the radius of the particle, and  $\gamma$  the concentration of univalent electrolyte. Increased concentration or charges on ions tend to make the minimum deeper and reduce its distance as shown in Text-fig. 13. At some critical concentration the minimum will move to where the particles are in contact, but at this point Van der Waals forces will play a predominating rôle. Now in most cases with spherical particles the depth of this energy minimum is smaller than the thermal energy  $kT$  for the particles, consequently they will behave like gas molecules interacting for short periods, and only in the presence of strong salt solutions are they likely to come together and coalesce in crystals or amorphous precipitates.

The situation for long particles, however, is very different. Levine has so far treated only the case of two long cylindrical particles arranged parallel to each other. It is easy to see that the type of mutual energy curve remains unchanged. But the depth of the energy minimum is for particles of length more than about ten times their thickness, far greater than  $kT$  at ordinary temperatures. The result is that here stable equilibrium will occur with a mutual distance depending on the ionic concentration. Methodical treatment so far has only been carried out for parallel particles, but it is easy to see that any displacements from parallelism would, in the case of long particles meet with considerable restoring forces. The energy minimum will occur always in the parallel position and the relative depth of the minimum will be greater the longer the particles. As long as  $kT$  for libration is less than a minimum value determined by the length distance ratio, stable equilibrium will result. When it is of the same order, as will occur at high dilutions or with short particles, the libration disturbance will cause the particles to break apart at a distance less than that where they would still be stable for simple lateral displacement. What applies to two particles holds *a fortiori* for a large number of particles. If the energy minima are deep enough, they must arrange themselves in a regular hexagonal lattice, just as they are observed to do in the virus. The mathematical treatment of the equilibrium in this case has yet to be carried out, but it is easy to see that the result will only lead to a slight modification of the equilibrium position in the case of two long particles, the effects of the other particles resulting in a somewhat greater equilibrium distance and lower mutual energy. Levine's theory can therefore be tested directly by the measurements made on the relation of interparticle distance to ionic concentrations for virus particles, and Text-fig. 14 shows how good the agreement is in the shape of curves. Absolute comparisons are impossible at this stage of the theory as computations have not been made for sufficiently high concentrations nor has the character of the tobacco mosaic virus been taken into consideration.

We may, however, say that we have now an adequate semiquantitative explanation of the unexpected phenomena of the regularity observed in the

virus, and it is also clear that this has a far wider application, namely to all fibrous colloid molecules (see p. 145). The arrangement of long particles in equilibrium in a medium furnishes us with a new type of arrangement of matter, namely that of an elastic two dimensional crystalline solution. If a small amount of solvent is present the particles are near together, when the solvent is added they move apart to fill the space, but not through irregular movements as in a gas, but by regular expansion of the particle lattice. It is



TEXT-FIG. 14. Experimental relation between interparticle equilibrium distance and Levine's function.

probable that most phenomena of swelling have the same character. Where platy particles are involved the equilibrium can be maintained at far greater distances than with cylindrical ones, owing to the fact that ion concentration falls off in a linear manner instead of roughly as inverse square of the distance.

In fact in this case the distances reach 5000 Å or more and the phenomenon becomes visible as in iridescent sol.

#### *Hydration of the Particles*

It can be seen from Text-fig. 10 that in strong salt solutions the distance between the particles shrinks to a steady value of 173 Å, but that this value is distinctly greater than that between the particles in the dry state. This is not as yet fully accounted for by Levine's theory. It would appear that the

model taken for the theory is too simple in that it does not allow for the finite molecular structure of the water medium. The differences between the two values indicates that there is a layer of some 20 Å in thickness between the particles in the most concentrated solutions. This would correspond to one of 10 Å surrounding the particle or roughly layers of four water molecules. We may consider this water to be bound not by the total charge of the particle but by the positive and negative charges due to amino and carboxy groups which cover it even at the isoelectric point. If this is so, it should be possible to demonstrate the attachment of the water by physical methods, but so far the amounts of material available have not been sufficient to do this. It is the presence of this closely bound water that gave rise, at an earlier stage of the investigation, to the belief in two separate phases, wet gel and dry gel; and indeed the layer may represent one of particular stability as it has up till now proved impossible to prepare specimens with an intermolecular distance of between 159 Å and 169 Å.

#### *Equilibrium between Top and Bottom Layers*

The relation between interparticle distance and ionic concentrations can be followed by direct measurement only down to concentrations of 13 per cent, that is to an interparticle distance of 450 Å. At greater dilutions it is clear that equilibrium still exists, the presence of the order disorder phases is enough to show that. Further, the dilution shows that the equilibrium occurs at much greater distances. The attempt, however, to follow the equilibrium by observing the concentrations of top and bottom layers in equilibrium in dilute salt solution led to paradoxical results. Only very small amounts of material were available at this stage and therefore the measurements are too rough to be worth quoting but their trend is unmistakable. On adding dilute salt solution to an equilibrium mixture of top and bottom solutions, the level of separation moves upward, that is the addition of salt solution increases the critical dilution and hence the distance between the particles at equilibrium. When the concentration of the salt solution exceeds a definite value roughly  $\tau = 16$ , separation of top and bottom solution can no longer be observed. Instead first elongated tactoids and then fibres are formed (Plate 2, Figs. 11–14).

An explanation of this paradoxical behaviour is to be sought in studying more closely the actual conditions of equilibrium of the particles in more dilute solutions. It is here that the tactoid formation, already described above (p. 128), occurs, and consideration of this shows that the phenomenon in question is one which involves the length of the particles.

#### *Theory of Tactoid Form*

The tactoid form is characteristic of a liquid crystal of the type formed by the virus particles, that is, of one of long thin particles arranged parallel and

equidistant to each other. This type has not hitherto been described but it corresponds to the class *RRD* in Hermann's (1931) classification of possible liquid crystal systems. Tactoids bear a superficial resemblance to the baton-net of smectic liquid crystals (Friedel and Friedel, 1931), but these have radically different structures, being composed of coaxial cylinders of molecular sheets in which the molecules are perpendicular and not parallel to the axis of elongation.

On the surface of a tactoid the particles of the ordered phase are arranged parallel to each other and parallel to the meridian lines of the tactoids. This follows from direct observation of their birefringence. Now when long particles are involved, the surface of separation can no longer possess the isotropic character of an ordinary liquid interface. The bending of such a surface must require different amounts of work according to whether the bending takes place along the axis parallel to or perpendicular to the orientation of the particles in the surface. *Such a surface will in fact behave as if it possessed an anisotropic interfacial tension.* We may consider such a surface as one possessing two principal surface tensions,  $\sigma_1$  and  $\sigma_2$ , the first being in general greater than the second, their ratio depending not necessarily in any simple way on the relative length to breadth of the particles. The simplest expression for an equilibrium surface would in this case be

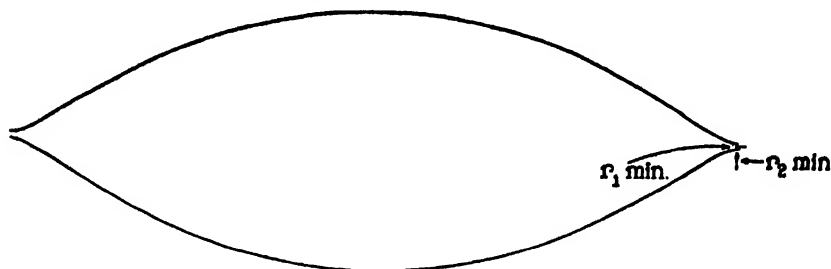
$$\frac{\sigma_1}{r_1} + \frac{\sigma_2}{r_2} = \text{const.}$$

where  $r_1$  and  $r_2$  are the two principal radii of curvature of the surface. Corresponding to a simpler expression for an ordinary interface

$$\sigma \left( \frac{1}{r_1} + \frac{1}{r_2} \right) = \text{constant}$$

This condition is not, however, sufficient to define the surface of the tactoid. It is clearly also necessary that the surface energy for a given volume must be a minimum and the mathematical formulation for this has not yet been found. It is, however, easy to determine the general character of the surface and to describe it approximately graphically (Text-fig. 15). It is a surface of revolution generated by the curve shown in Text-fig. 15. Its curvature is determined by  $r_1$  while  $r_2$  is given by the part of the normal intercepted by the axis of revolution. The minimum positive value of  $r_1$  occurs at the equatorial plane. As  $r_2$  diminishes  $r_1$  increases and passing through an infinite value at a point of inflection, becomes negative, and reaches a minimum negative value where the curve is again parallel to the axis, after which the curve repeats indefinitely. The general shape of the surface is therefore that of a series of barrel-shaped portions separated by hour glass-shaped constrictions. These constrictions, however, in cases where  $\sigma_1 > \sigma_2$  almost reduce to cusps and the

anomalous arrangement at these points leads probably to their being occupied by particles of impurities.<sup>5</sup> Where  $\sigma_1$  is nearly equal to  $\sigma_2$  the shape approaches the spherical, where  $\sigma_1$  is much greater it is long and tapering, approaching a fibre-like structure which is observed in old gels and in the fibres studied by Best (1937 *a* and *b*). The shape is dependent not only on the ratio of  $\sigma_1$  to  $\sigma_2$  for, unlike the case of a normal liquid, the shape of a tactoid drop also depends on its size. The relation must be complicated but broadly it may be stated that the larger the tactoid the more it resembles a sphere, the smaller the more it resembles a cylindrical or needle-shaped body. How well this geometrical theory fits the observations is shown in Plate 1, Fig. 3, where a number of tactoids in equilibrium can be seen and compared to the theoretical curves. The extreme case is that of the acicular micro-tactoids formed by acid or salt precipitations first described as crystals by Stanley (1935), (1937).



TEXT-FIG. 15. Theoretical axial cross-section for tactoid surface. (The subscripts of  $r_1$  and  $r_2$  should be interchanged.)

The gradations between normal tactoids and fibres can well be seen in the series of preparations with different ionic concentrations as shown in Plate 2, Figs. 11–14. Those containing more salt show more elongated tactoids and a greater tendency to fibre formation. The radius of curvature changes from 0.07 mm. in salt-free solutions to 0.12 mm. in 0.1 N solutions. It is clear that the condition of stability of a tactoid depends on two factors, the length of the particles, and the degree to which mutual influence between the particles maintains parallelism between them. Although it has not yet been calculated the orienting force can be seen to depend according to Levine's theory on the ratio of length to distance apart of the particles. If two particles of finite length be imagined to move away from each other, it is clear that the steepness of the potential trough for angular displacements will diminish, and diminish faster the shorter the particles. For concentrated solutions where the ratio of length of particles to mean distance apart is very great, orientation will

<sup>5</sup> That this is probable is shown by the extrusion of an intermediate layer of impurities on the settling of bottom layer solution, that is, on increasing the size and diminishing the number of independent tactoids.

be perfect, but as the salt concentration falls off the point will be reached when the system will become unstable, not owing to the transverse motion of the particles, but due to their angular oscillation. For particles of finite length, therefore, equilibrium will be reached at greater dilution the longer the particles and the higher the concentration of ions in the solution. This relation will hold until the concentration of ions is so great that particles cannot separate due to oscillatory movements alone. This is the explanation of the apparently anomalous behaviour of weak solutions described below. For different lengths of particles the behaviour at low concentrations will be different, but all will have the same behavior at high concentrations and for every particle length there will be a critical concentration at which the maximum interparticle distance is obtained.

#### *Equilibrium Concentrations of Top and Bottom Layer*

The variation of equilibrium concentrations with length of particle is very well shown in the study by Bawden and Pirie (1937*a*) on the effect of purification of the virus. One effect of this purification would be to separate the longer from the shorter particles. The first method used was to allow the virus solution to separate into two layers, to take the bottom layer and to dilute it with water up to the original volume and to allow further separation. If the virus originally contained particles of different lengths in equilibrium there would be a greater concentration of shorter particles in the top layer and the process described would at each stage lead to more uniform and longer particles in the bottom layer. The second method of purification, by means of trypsin digestion also probably led to longer particles, possibly by removing material at the end of the particles which prevented them joining with others. The general observation of Bawden and Pirie was that further purification of the virus altered very markedly the equilibrium concentration between top and bottom layer solutions.

Unpurified virus, *i.e.* salt-precipitated but not subjected to the above methods, remained in the top layer solutions up to concentrations of 4 per cent, when it was in equilibrium with bottom solutions of concentrations of 8 per cent, while highly purified solutions of as little as 1.6 per cent divided into two layers, the difference of concentration between them being as low as 0.02 per cent. Moreover, at this concentration, the top solutions showed a flow birefringence which remained in evidence for some seconds after actual flow had ceased. This suggests that we are dealing with an equilibrium system in which the alternation of one variable causes a continuous transition between a distinct two phase region and a critical condition in which only one phase is possible. One plausible suggestion is that the length of the particles is the variable factor though the effect of unidentified impurities may be of as great or greater importance.

Consider the conditions that determine the limiting concentration in top and bottom layer solutions. In the top layer, this is fixed by the limit of the space available to a particle for free movement. The minimum space may be taken as that of a flat cylinder of height equal to the diameter of the particle ( $d$ ) and diameter equal to its length ( $l$ ) (see p. 143). Its volume is accordingly

$$\frac{\pi^2 d}{4}$$

It may actually be smaller than this if particles on moving around manage to clear others out of their way without jamming, but it is difficult to work this out theoretically. In the bottom layer on the other hand, it is reasonable to suppose that the equilibrium distance between parallel particles is a simple function of their length, that is, that the possibility of maintaining a parallel alignment is a function of the ratio of the distance ( $r$ ) of the particles apart to their length, and that beyond a certain critical ratio ( $K$ ) brownian movement would make the arrangement unstable. On the simplest hypothesis, therefore, the volume occupied by a single particle will be:

$$\frac{\sqrt{3}}{2} r l = \frac{\sqrt{3}}{2} K^2 l^2 \quad \text{where} \quad K = \frac{r}{l}$$

The limiting concentrations of the top and bottom layers are therefore  $\frac{4m}{\pi l d}$  and  $\frac{2m}{\sqrt{3} K^2 l^2}$  respectively where  $m$  is the mass of dry virus per unit length.

It can be seen that as the length of the particle increases both equilibrium concentrations decrease, and further that at a certain critical concentration the difference between them vanishes, that is where

$$l = \frac{\pi d}{2\sqrt{3} K^2}$$

This concentration appears to be 1.8 per cent for tobacco mosaic virus (Bawden and Pirie, 1937 *a*).<sup>6</sup> For a solution of twice this concentration in the top layer, 3.6 per cent, the corresponding bottom layer concentration would be four times, or 7.2 per cent which approximates more closely than should be expected with the values 4 per cent and 8 per cent respectively given by Bawden and Pirie. Moving to higher concentrations we find that a limiting concentration of 9 per cent in the top layer would correspond to one of 45 per cent for the bottom, which is a maximum concentration, being approximately that of wet gel or precipitated crystals. This suggests that the phenomena of separation

<sup>6</sup> Page 287.

into layers, and indeed the existence of bottom layer, can only be observed for particles whose lengths are not less than one-fifth of the critical length.

From the recent measurements of Kausche *et al.* (1939) on tobacco mosaic virus we find this ratio is at least ten to one, so that we may expect orientation always to occur for tobacco mosaic virus at sufficient concentrations. On the other hand the conditions for observing it for other viruses whose particles are not so long though still anisotropic may be more difficult to achieve. It should be possible to check this theory quantitatively when samples of virus of uniform and known length can be obtained, and further to check it by measuring the effect of ionic concentrations and pH on its equilibrium. The influence of temperature on equilibrium would give further information, particularly of the interaction energy. Preliminary measurements show that while changes of temperature affect the equilibrium in the expected way, that is rise of temperature diminishes the amount of the ordered phase, the effect is a small one and therefore corresponds to a very small interaction energy. The whole problem has now acquired such theoretical importance that it deserves an extended study of its own, using uniform and purified materials in large quantities.

### *The Mechanism of Gel Formation*

The conditions of equilibrium between elongated particles in ionic solutions has a very close bearing on the formation of gels. A simple experiment illustrates this in the case of tobacco mosaic virus. If a dilute solution in the oriented phase, of concentration about 4 per cent, is flowed through a capillary tube it behaves like a uniform uniaxial crystal (Text-fig. 11 *a*). If now some acid is introduced in one end of the tube, then as long as the flow continues the same appearance is maintained and there is no notable change in the viscosity. We know, however, from the x-ray study that now the particles are very unevenly distributed and that the liquid is really a two phase one consisting of long tactoids with about 50 per cent dry weight of virus, and of almost pure solvent (Text-fig. 11 *b*). Once the flow is stopped particularly if the tube is jerked, the orientation largely vanishes and the liquid changes to a fairly rigid gel. It is clear that here disturbance of flow lines causes different tactoids to touch and form a combined tactoid framework throughout the liquid. We know that two tactoids in contact fuse into a branched tactoid (see p. 128). We may therefore consider that the whole of the more concentrated phase is a single tactoid (Text-fig. 11 *c*). Now the behaviour described is typical of a rheopectic gel, and it would seem very probable that a similar explanation holds for all cases of gel formation, in which long thin particles are involved. In such cases a gel must be considered to be a two phase system in which one of the phases is a solvent containing a small proportion of particles and the other is a liquid crystalline branched aggregate also containing a



variable amount of the solvent. On standing the aggregation may go further and the gel breaks down into microscopic fibres. Other gels, particularly those showing thixotropic characters, are probably formed under analogous but somewhat different conditions on account of the different nature of the forces between flat and long particles. This type of explanation would seem to offer a solution to the controversy between the rival theories of contact of particles and long distance forces in the explanation of gel formation. Both theories would seem to be right, though in a different sense than that originally propounded. The long distance forces are not exercised through the main bulk of the gel, but only between particles constituting individual tactoids; whereas the interaction between tactoids corresponds to the supposed contacts of colloidal particles. At the present stage, it is only possible to say safely that this applies to tobacco mosaic virus, but the methods of investigation described above could well be used to check the validity of this theory for other colloidal systems.

The Summary and Bibliography appear at the end of Part III.

## EXPLANATION OF PLATES

### PLATE 1

Photographs of orientation phenomena of virus solutions and gels (crossed nicols).

FIG. 1. Tobacco mosaic virus solution in drying cell showing (a) bottom layer solution; (b) wet gel; and (c) dry gel.

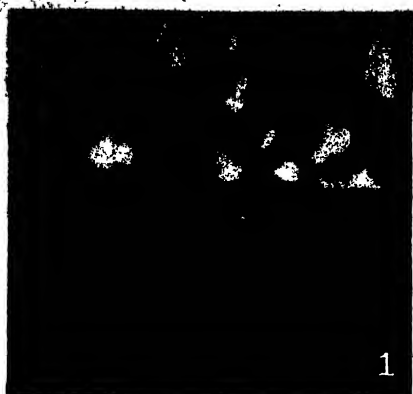
FIG. 2. As Fig. 1, but showing (a) flow of bottom layer solution around end of spacer rod from left to right and (b) zigzag drying in wet gel.

FIG. 3. "Positive" tactoids of bottom layer solution in top layer.

FIG. 4. "Negative" tactoids of top layer in bottom layer solution showing sinuous orientation directions.

FIG. 5. Oriented bottom layer solution in capillary tube with quartz wedge showing compensation.

FIG. 6. Orientation of top layer solution by alternating current showing electrodes and luminous patch of oriented top layer.



a  
b  
c

1



a  
b

2



4



3



5



6

## PLATE 2

Further orientation phenomena of virus solutions and gels.

Reversed spiral structures Figs. 7-9.

FIG. 7. Reversed spirals in wide tube. Taken by Professor Van Iterson. (Crossed nicols.)

FIG. 8. Reversed spirals in narrow tube. Taken by Professor Van Iterson. (Crossed nicols.) Showing dark—parallel oriented and light—spirally arranged portions.

FIG. 9. With one nicol rotated showing alternate sections with same direction of rotation of polarisation.

FIG. 10. Zigzag structure of virus dried on plate (unpolarised light) taken by Professor Van Iterson.

Effects of dilute salt solutions on virus preparations—Figs. 11-14.

FIG. 11. Four capillaries with the same virus concentration but with salt content increasing from right to left. Note the greater amount of bottom solution as salt content increases (crossed nicols).

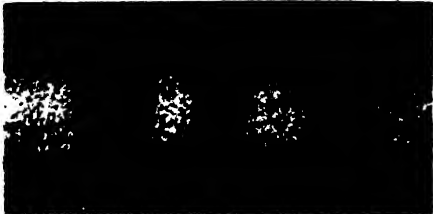
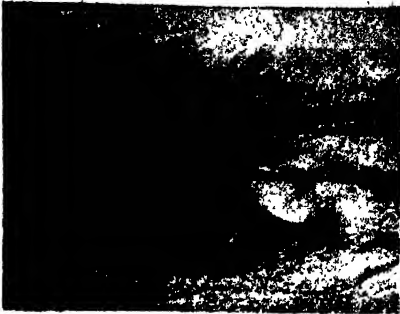
FIG. 12. 0.1 N  $(\text{NH}_4)_2\text{SO}_4$  solution showing negative tactoids (crossed nicols).

FIG. 13. 0.2 N  $(\text{NH}_4)_2\text{SO}_4$  solution showing elongated negative tactoids (crossed nicols).

FIG. 14. 0.3 N  $(\text{NH}_4)_2\text{SO}_4$  solution showing gel fibres (unpolarised light).



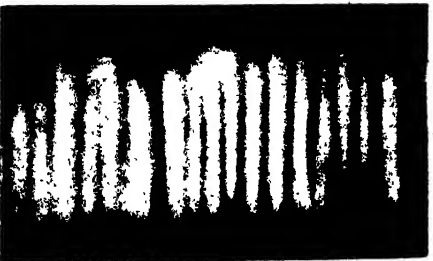
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9



8



7



### PLATE 3

Inter- and intramolecular x-ray photographs of various virus preparations.

Intermolecular photographs—Figs. 15–18.

FIG. 15. Showing shift of intermolecular reflections on passing from (lowest film) dry gel to (middle film) wet gel and (uppermost film) 1.3 per cent solution.

FIG. 16. Effect of concentration of virus on spacing.

- (a) 1.3 per cent solution.      Unfiltered Cu radiation
- (b) 2.3 per cent solution.      Unfiltered Cu radiation
- (c) Top half, 3.4 per cent solution.      Unfiltered Cu radiation  
Bottom half, 3.4 per cent solution.      Filtered Cu radiation
- (d) 3.4 per cent solution.      Monochromatic Cu  $K_{\alpha}$  radiation
- (e) 5.0 per cent gel.      Unfiltered Cu radiation

FIG. 17. Enation mosaic virus solution photographed with monochromatic Cu  $K_{\alpha}$  radiation showing extremely sharp lines due to perfect orientation and arrangement.

FIG. 18. Intermolecular pattern of dry gels of different virus proteins. (a) tobacco mosaic virus. (b) enation. (c) aucuba. (d) cucumber.

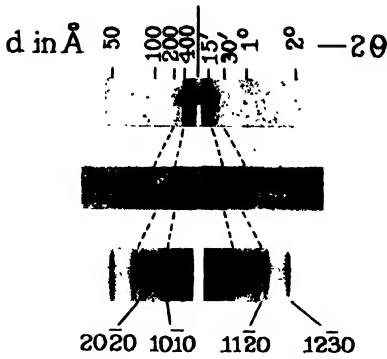
Plate distances: Figs. 15, 16, 17 —40 cm.; Fig. 18—16 cm.

Intramolecular photographs—Figs. 19–21.

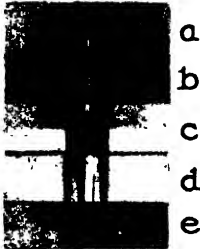
FIG. 19. Typical early dry gel photograph of tobacco mosaic virus.

FIG. 20. Cucumber mosaic virus.

FIG. 21. Potato virus X (shorter plate distance). Note the two lines 2 and 4 inside the strong meridian line 6 instead of the single line 3 on tobacco and cucumber virus photographs.



15

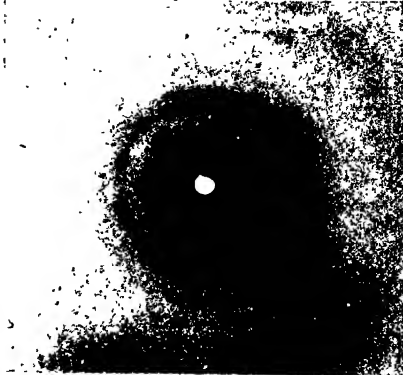


16

17



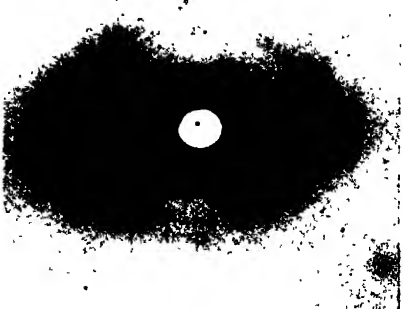
18



19



20



21

#### PLATE 4

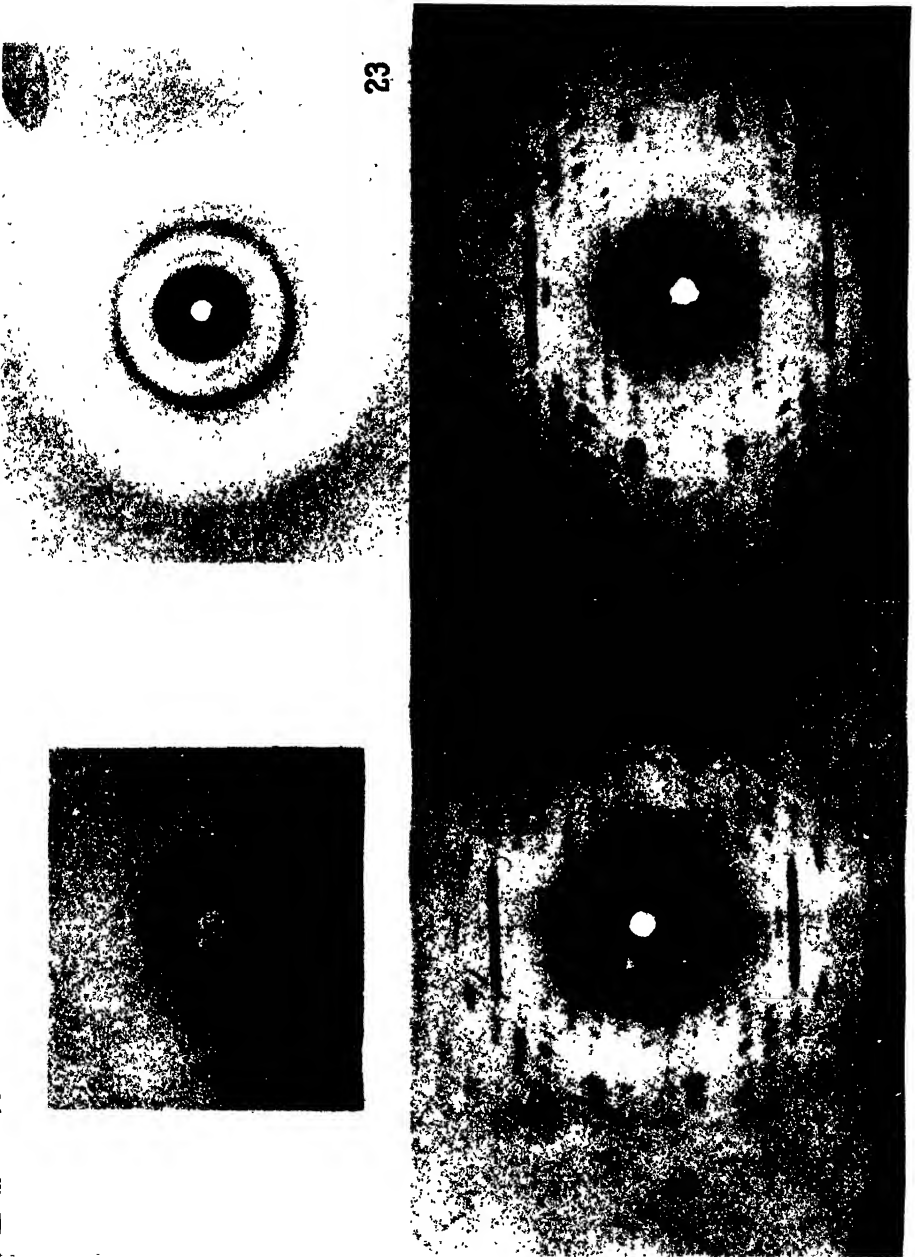
##### X-ray photographs of tobacco mosaic virus.

FIG. 22. Intermolecular pin-hole photograph (plate distance 30 cm.) of small piece of dry gel mounted with x-rays along particle direction showing orientation relative to surface (vertical in photograph) and hexagonal single crystal character. It can be seen that the  $(11\bar{2}0)$  and not the  $(1\bar{1}00)$  planes lie parallel to the surface (air-water interface in drying cell (Text-fig 2)).

FIG. 23. Intramolecular pin-hole photograph (plate distance 6 cm.) of the same piece showing completely random orientation of individual particles.

FIG. 24. Intramolecular photograph of 36 per cent enation solution in capillary tube (direct print). (Plate distance 14.8 cm.)

FIG. 25. Same as Fig. 24, but printed through a rotating sector disc to show all important reflections of comparable intensity.



(Bernal and Fankuchen: X-ray and crystallographic studies of plant virus. I, II)





# X-RAY AND CRYSTALLOGRAPHIC STUDIES OF PLANT VIRUS PREPARATIONS. III

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## 1. The Structure of Particles

Almost the whole of our evidence for the internal structure of the particles is derived from x-ray observations, particularly those of the high angle or intramolecular photographs. In contradistinction to the low angle photographs, the most striking feature is that the general appearance of the pattern is independent of the state of hydration.

Most of the photographs have been taken from oriented specimens. This is because on unoriented specimens the wealth of lines makes direct interpretation impossible, though with a knowledge of the oriented pattern they may all be identified. Table IV shows the close correspondence between the spacings of the spots on our oriented photographs (Plate 4, Fig. 25)<sup>1</sup> with the rings on the unoriented photographs of Wyckoff and Corey (1936). The oriented specimens gave typical fibre photographs. In the case of the large two dimensional crystals of the dry gel, which possess secondary orientation relative to the water surface, a careful search was made to see if the large-angle scattering also showed such orientation, but no trace of it was found (Plate 4, Fig. 23). It would therefore appear that the only mutual orientation which affects the inner portions of the particles is that of parallelism of their long axes. But this negative result may be due to the disorientation of the dry gel on shrinking.

The large-angle scattering of the various virus preparations shows characteristic similarities and differences. Only four distinct patterns have been observed. The first, which has been most carefully studied, is common to all the strains of tobacco mosaic virus, a second barely distinguishable from it is given by the cucumber virus (Plate 3, Fig. 20). A third is given by both strains of the potato virus X (Plate 3, Fig. 21). The fourth pattern, which is difficult to compare with the others owing to the absence of orientation, is given by the isotropic bushy stunt virus of tomato (Bernal, Fankuchen, and Riley, 1938).

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<sup>1</sup> The plates appear at the end of Parts I and II.

### *The Intramolecular Pattern*

Of these only the pattern of the tobacco mosaic strains has been examined, in any detail, though enough is known of the others to show their general relationships. Early photographs of the tobacco mosaic virus, as either oriented gel or as liquid, gave the pattern shown in Plate 3, Fig. 19, containing four main spots (0003), (0006), (4482), and (3037). Of these the (4482), (0006), and (3037), lay approximately on a Debye circle of 11 Å, the region of strong reflection for proteins. This and indications of another ring of reflections at about 4.5 Å were sufficient to show that the picture was of the same type as those of proteins. The sharpness of the spots was, however, definitely greater than for the natural fibre proteins, except perhaps feather keratin. They were not, however, at this stage by any means as sharp as those from crystalline proteins (Bernal, Fankuchen, and Perutz, 1938). Further studies, however, showed that this was largely due to lack of orientation. It was found that the clearest photographs could be obtained from solutions oriented in capillary tubes and after many trials remarkably perfect photographs were obtained. The best (Plate 4, Figs. 24 and 25) which required an exposure of nearly 400 hours, showed hundreds of distinct spots some of which, particularly near the meridian line, were as sharp as those from crystalline proteins.

### *Interpretation*

The interpretation of the intramolecular pattern seemed at first sight fairly straightforward. It corresponded to that of a crystal with a hexagonal lattice of  $a = 87$  Å,  $c = 68$  Å (Bernal, 1938). As progressively better photographs were obtained several spots appeared which could not be explained by such a small cell. It was hoped that by obtaining still better photographs this difficulty could be cleared up, but the best obtained merely confirmed the impossibility of the simple interpretation. Orthorhombic and monoclinic pseudo-hexagonal cells were next tried, but they also failed to account satisfactorily for the observed reflections, while calling for many others that were not observed. It appeared that it was quite impossible to explain the pattern on the existing theory of x-ray diffraction from a crystal, for any cell large enough to give the observed spots was found to be larger than the size of the particle as inferred from all the intermolecular measurements.

The key to what may be the true explanation of this paradox, and what is in any case a plausible one, was found in a detailed comparison of the intramolecular photographs of the dry gels, wet gels, and solutions. The equatorial line offers the greatest interest, for here the intermolecular pattern of the dry gel is found to pass almost continuously into the near intramolecular pattern of the solutions, as shown in Tables I and IV. Particularly significant are the two reflections at  $\xi$  values of approximately 0.031 and 0.043,<sup>2</sup> (corre-

<sup>2</sup>  $\xi$  is a coordinate of a reflecting plane in reciprocal space. For details see Bernal (1927).

TABLE I a

*Intermolecular Data from Air Dried Anisotropic Gels. Relative Intensities, Spacings, and Interparticle Distances in Angstrom Units*

$h^2 + k^2 + l^2$	<i>hkl</i> Hexagonal indices	Spacing		Tobacco mosaic virus			Cucumber virus		Potato virus X
		Observed	Calculated	Ordinary	Enation	Aucuba	Cucumber 3	Cucumber 4	
1	1010	131.8	131.8	<i>s</i> +	<i>w</i> - <i>m</i>	<i>m</i>	<i>m</i>	<i>w</i>	No intermolecular reflections observed
3	1120	75.8	76.0	<i>s</i>	<i>s</i>	<i>s</i>	<i>m</i>	<i>w</i> - <i>m</i>	
4	2020	65.9	65.9	<i>m</i>	<i>m</i>	<i>w</i> - <i>m</i>	<i>w</i>	<i>vw</i>	
7	2130	49.8		<i>m</i> - <i>s</i>	<i>m</i> - <i>s</i>	<i>m</i> - <i>s</i>	<i>s</i>	<i>s</i>	
9	3030	43.5	43.9	<i>vw</i>	0	0	0	0	
12	2240		37.3						
13	3140		36.7	0	<i>vw</i>	0	<i>m</i> - <i>s</i>	<i>m</i>	
Interparticle distance . . . . .				152	152	152	146	146	

The intensities are very sensitive to slight variations of water content. It has been observed for tobacco mosaic virus that the presence of moisture tends to weaken the (1010) reflection and to enhance the (2240) and (3140).

TABLE I b

*X-Ray Data from Bushy Stunt Virus*

	Wet crystals	Dry crystals
Side of unit cell....	394 Å	316 Å
Cell molecular weight.....	48,000,000	25,600,000

Reflections observed					
hkl	Observed		hkl	Observed	
	Spacing	Intensity		Spacing	Intensity
110	279	ws	110	224	w
112	160	w - m			
220	140	vw			
310	129	vw			
440)	70-67	vw			
600)					
660	47	vw			
880	35	vw			
888	28.5	vw			

The unit cell of the wet crystal is body centered cubic and contains two molecules. Despite the fact that only one reflection was observed from the dry crystals, there are good reasons for assigning to it the same type unit cell.

TABLE II

*Data Illustrating the Relation between Spacing and Concentration in Virus Preparations*

N = No. of grams vacuum-dried protein in 100 cc. of specimen.

T.M.V. = tobacco mosaic virus.

Virus used	N	Spacing observed d(1010)	Interparticle distance	$\frac{1650}{\sqrt{N}}$
T.M.V. vacuum dried.....	137	129	149	143
T.M.V. enation, and aucuba, air dried.....	131	132	152	144
T.M.V. wet gel.....	57.5	184-192	213-222	217
Aucuba.....	34	252	291	281
Enation.....	26	259	300	324
Enation.....	25	287	332	330
T.M.V.....	23	297	343	340
T.M.V.....	13	397	458	460

TABLE III

*The Dependence of Interparticle Distance in Equilibrium Gels on Salt Concentration and pH*

## (a) Gels in ammonium sulfate solution

Normality of $(\text{NH}_4)_2\text{SO}_4$	d(1010)	Interparticle distance
8.25	150	173
6.2	150	173
4.12	158	182
2.06	161	186
1.03	182	210
0.52	192	223
0.33	198	229
0.26	216-261	251-302

## (b) Gels at varying pH

pH	d(1010)	Interparticle distance
2.5	Specimen dispersed	
3.0	171	198
3.4	158	182
3.4	160	185
3.4	167	192
4.0	174	201
5.0	206	237
6.0	252	289
7.0	~263	~305

spanding to spacings of 50 and 36 Å respectively). In the dry gel they correspond to the reflections of (1230) and the doublet (2240 and 1340); in the 50 per cent wet gel to (1340) and (5050). Beyond this they cannot be clearly

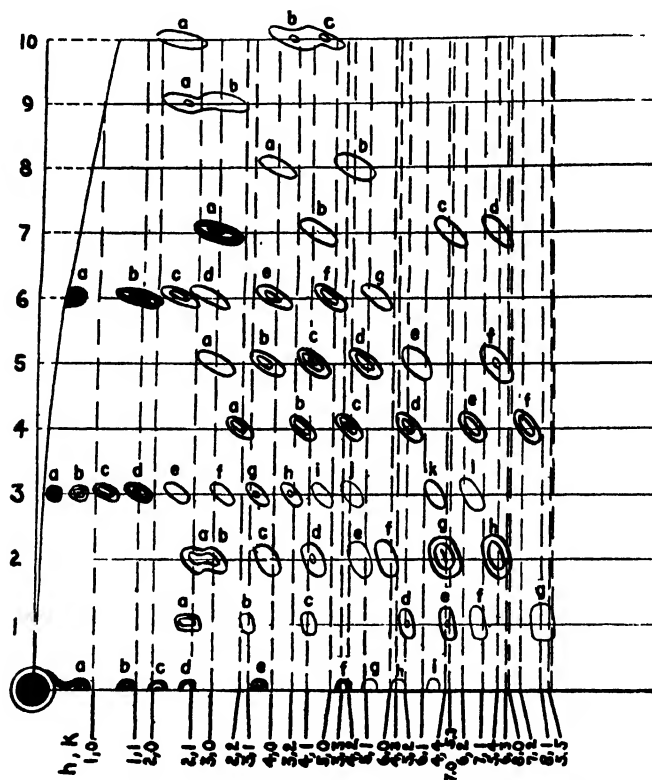
identified with any intermolecular spacing, but their position has hardly changed. There can be no reasonable doubt that they correspond to impor-

TABLE IV  
*Intramolecular data on tobacco mosaic virus*

Powder data (Wyckoff and Corey)					Outstanding intramolecular reflections from best oriented specimen (enation)				
Ordinary strain		Aucuba strain		Ultra-violet irradiated spacing	Spacing in Å	Layer line	Letter for reflection Text-fig. 16	Indices in terms of hexagonal unit cell $a = 88 \text{ Å}$	Intensity
Spacing	Intensity	Spacing	Intensity						
80	<i>s</i>	80	<i>s</i>		93	0	a	Fresnel scattering	<i>s</i>
53	<i>m</i>	55	<i>m</i>		47	0	b	(4/3, 1/3, 5/3, 0)	<i>s</i>
37	<i>w</i>	37	<i>w</i>		36	0	c	(5/3, 2/3, 7/3, 0)	<i>s</i>
28	<i>vw</i>	27	<i>vw</i>		29	0	d	(7/3, 1/3, 8/3, 0)	<i>m</i>
					19.7	0	e	(8/3, 5/3, 13/3, 0)	<i>m - s</i>
20.8	<i>m</i>	21.0	<i>m</i>	21.2	22.0	2	a	(2, 1, 3, 2)	<i>m</i>
					23.0	3	a	(0, 0, 0, 3)	<i>vs</i>
					22.3	3	b	(1/3, 1/3, 2/3, 3)	<i>m - s</i>
16.2 } 14.2 }	<i>vw</i>	14.2	<i>vw</i>	14.7	Too many reflections of this spacing to list (see Plate 4, Fig. 25, and Text-fig. 16).				
11.0	<i>s</i>	11.0	<i>s</i>	11.3	11.4	6	a	(0006)	<i>vs</i>
10.2	<i>w</i>	10.2	<i>w</i>		10.3	2	g	(4452)	<i>m - s</i>
9.2	<i>m</i>	9.2	<i>m</i>	9.2	9.1	7	a	(3037)	<i>s</i>
7.44	<i>m</i>	7.43	<i>m</i>	7.5	For each reflection shorter than 20.8 Å there are too many intramolecular reflections corresponding to it in spacing for the correlations to have any special significance. The intense reflections corresponding to 11.0, 10.2, 9.2 Å, are given merely because these are strong lines and the reflections principally responsible for them can be determined.				
6.5	<i>w</i>	6.5	<i>w</i>						
5.7	<i>vw</i>								
5.44	<i>w</i>	5.41	<i>w</i>						
4.95	<i>m</i>	4.95	<i>m</i>						
4.71	<i>m</i>	4.68	<i>m</i>						
4.44	<i>m</i>	4.45	<i>m</i>	4.46					
4.08	<i>w</i>	4.09	<i>w</i>	4.10					
3.88	<i>m</i>	3.88	<i>m</i>	3.88					
3.70	<i>vw</i>								
3.54	<i>vw</i>								
3.39	<i>w</i>	3.39	<i>w</i>						

tant intramolecular reflections which serve to enhance the intensity of the intermolecular reflections lying nearest them. The spot on the third layer line which corresponds to them is a single one at  $\xi = 0.036$ , which would be (3033) for the dry gel and remains unchanged in position, within errors of measurement, for the other forms.

Now the fact that these intramolecular values though arrived at quite independently fit with intermolecular ones for the dry gel points to the not unexpected conclusion that its intermolecular periodicity is closely linked to the intramolecular one. We cannot, however, attempt to index the intramolecular pattern on this basis for that would bring us back to the paradox that the cell



TEXT-FIG. 16. Projection of reciprocal net of hexagonal unit cell to show indexing of reflections of Plate 4, Fig. 24.  $a = 87 \text{ \AA}$ ,  $c = 68 \text{ \AA}$ . Region between  $y$  axis and curved line is a forbidden zone (Bernal, 1927).

size would be larger than the particle, but we can make the complementary assumption that for the scattering of the isolated virus particle referred to a true repeat unit inside the particle, fractional as well as integral indices are admissible for the reflecting planes. The assumption of fractional indices, daring as it seems, might but for its novelty have been logically deduced from the scattering conditions of the isolated particle. The Bragg theory of diffraction of x-rays by crystals is based on the assumption that the repeat unit is repeated an indefinite number of times. If this is not the case the subsidiary diffraction maxima can no longer be neglected. The theory is here necessarily complicated

and has not up till now been attempted largely because no known experimental examples of it existed. It is, however, in many respects similar to that of electron diffraction from very thin crystals. No attempt will be made here to develop it formally, merely to indicate the general manner in which the anomalous x-ray pattern of the virus particles may be explained.

The particle is short only in two dimensions. In the direction of its length there are many repeat units and therefore the diffraction effects corresponding to this direction are normal. In all other directions, however, fractional ( $hki$ ) indices are observed. Table IV and Text-fig. 16<sup>3</sup> show the allocation of these. Owing to the great number of possible planes for which  $\xi > 0.1$  and the intrinsic lack of sharpness in this region only the inner reflections can be indexed with certainty, and most of these occur on the equator and on the third and sixth layer lines. Some characteristic regularities can be noticed but have not as yet been explained. Thus, on the equator no reflections with integral indices appear. Reflections both with integral indices and fractional ones of the type ( $hh2hl$ ) occur on the third and sixth layer lines. The integral reflections here are strong and the fractional weak. It would appear as if each true reflection was forbidden on the equator and replaced by a doublet of fractional indices, while on the layer lines the true reflections appeared with two non-integral satellites forming a triplet. One equatorial reflection in particular, the strong reflection at  $\xi = 0.0165$  (93 Å) should be noticed as *it corresponds to the Fresnel scattering of the particle as a whole*. The second order interferences are found only for small values of  $\xi$  and here the uncertainty of measurement makes it difficult to identify them with certainty.

### Cell Size and Molecular Weight

If the explanation of the observed reflections given above be accepted, the apparent great complexity of the pattern is due to the diffraction effects not conforming with Bragg's law, owing to the finite size of the particles. It follows that in the discussion of the actual structure these effects can be disregarded in the first place, and an ideal repeat unit chosen which corresponds to the disposition of scattering matter inside each particle. This proves to be the cell originally chosen,  $a = 87$  Å,  $c = 68$  Å, which would provide exactly three cells in a hexagonal particle cross-section (Text-fig. 17). This is in excellent agreement with the particle diameter of  $150$  Å  $= 87\sqrt{3}$  derived from the intermolecular measurements on the dry gel. For such a cell assuming a density of 1.34 (Bawden and Pirie, 1937 *a*) the molecular weight would be 370,000. On account of the strength of the sixth layer line and the marked pseudorhombohedral character of the reflections, the true repeat units may well be one-sixth of this or 62,000. Assuming 6 per cent of this is nucleic acid this

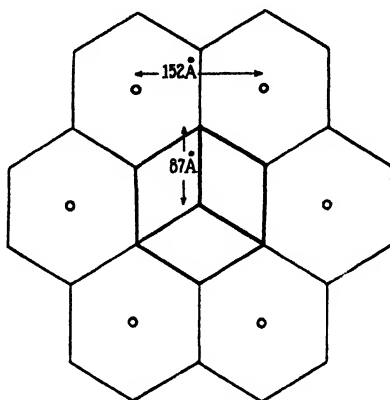
<sup>3</sup> The text-figures for Parts I, II, and III are numbered consecutively.



gives a molecular weight of 58,000, which is of the same order as that of a protein molecule, though it does not fit well into any of the Svedberg classes (Svedberg, 1939). It must be emphasized that this is a cell molecular weight and not the "molecular weight" of the gross virus particle which is, of course, very much greater.

### *Internal Structure*

It is difficult on the basis of the complicated x-ray evidence to go much deeper into the structure of the molecule. Certain deductions may, however, legitimately be made. The two most noticeable features of the pattern are first, the absence of strong reflections on the equator which shows that the particles

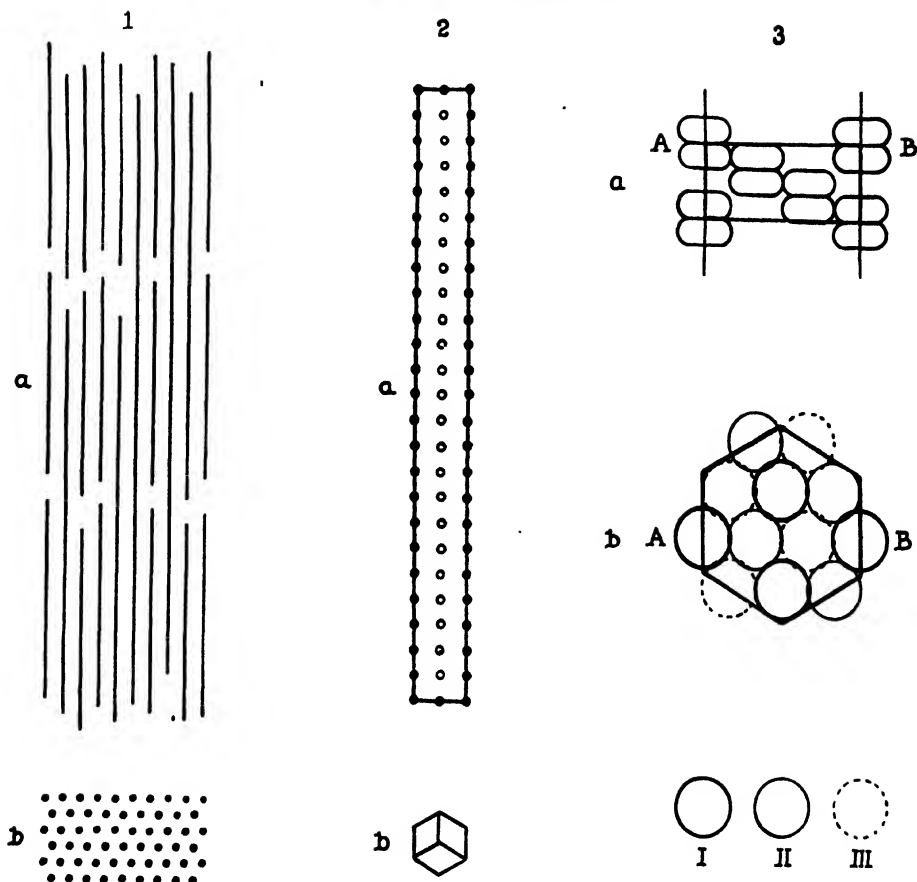


TEXT-FIG. 17. Diagrammatic cross-section of dry gel showing the relation of the unit cell of the particle with that of the interparticle packing.

cannot be composed of bundles of fibrous molecules and, second, the presence of extremely strong reflections perpendicular to the axes, indicating some form of layer structure. Further, the pattern is clearly differentiated into that of the zero, third, and sixth layer lines, and that of the other layer lines. This together with the absence of  $(10\bar{1}l)$  and  $(20\bar{2}l)$ , while  $(0003l)$  and  $(11\bar{2}l)$  are strongly represented, suggests a rhombohedral structure,  $a = 55 \text{ \AA}$ ,  $\alpha = 104\frac{1}{2}^\circ$ .

The presence of the planes  $(30\bar{3}2)$  and  $(31\bar{4}3)$  would appear to contradict this, but these may be anomalous reflections, or the structure may be only pseudorhombohedral with a triad screw axis.

Strong reflecting planes would appear to indicate sub-units; the larger sub-unit corresponding to the planes  $(0003)$  and  $(11\bar{2}l)$  would be a flat platelet of dimensions  $44 \times 44 \times 22 \text{ \AA}$ , the smaller corresponding to the strongest planes of  $4\bar{1}l$ ,  $(0006)$ , and  $(44\bar{8}2)$  and the ring of planes at spacings of about  $11 \text{ \AA}$  would be a roughly equidimensional sub-unit of diameter  $11 \text{ \AA}$ . The larger



TEXT-FIG. 18. Diagrams to illustrate tobacco mosaic virus structure seen at different magnifications. (In each case the view *above* is normal to the particle length, *below* along the particle length.)

1. Arrangement of particles in dry gel. *a* side view; *b* end view. 1 cm. = 600 Å.

2. Single virus particle showing subdivisions into crystal unit cells. ●—●—● indicates *c* axis repeat unit of 68 Å. *a* side view; *b* end view. 1 cm. = 200 Å.

3. Cubic close packing of large sub-units as seen in the three virus unit cells making up one particle. *a* side view; *b* end view. 1 cm. = 67 Å. *a* shows a section at AB of the view below (*b*). I, sub-unit at bottom of cell; II, sub-unit at  $\frac{2}{3}$  of unit cell height; III, sub-unit at  $\frac{1}{3}$  of unit cell height.

sub-units could be arranged in cubic close packing, as shown in Text-fig. 18. Further confirmation is furnished by the photographs of potato virus X (Plate 3, Fig. 21). Here we have the same strong reflection at 11 Å, but there are also reflections at 33 and 16.5 Å, suggesting that here also the 11 Å sub-units

are present but that the larger sub-units are  $44 \times 33 \text{ \AA}$  and are arranged in hexagonal rather than cubic close packing.

This is as far as it is safe to go on the basis of the experimental material available. There is no doubt, however, that with the help of more detailed photographs and comparisons with further virus types, it should be possible to obtain much more detailed information on the structure.

#### DISCUSSION

The evidence so far supports the view that the virus particle is in some sense intermediate between a molecule and a crystal of a normal crystalline protein. Its lateral dimensions are of the order of six times those of a protein molecule, while in the direction of its length, it contains a multiple of 24 true or 144 pseudocells, almost as many as a small protein crystal (Text-fig. 18 *b*). In detail the intramolecular pattern shows interesting resemblances and differences from those of the proteins that have hitherto been studied. Like them it shows the dominant 11 and 4.5  $\text{\AA}$  spacings, indicating the same basic internal structure (Bernal, 1939). It differs, however, from them by the fact that one of the 11  $\text{\AA}$  reflections, that perpendicular to the particle length predominates over all the others. This indicates either sheets or sets of parallel fibres with this lateral spacing, or, what comes to much the same thing, a high degree of alignment of the smallest sub-units. To a certain degree it resembles the fibrous and denatured proteins in this respect and may like them have a simpler structure than that of the crystalline proteins.<sup>4</sup> Such a deduction would fit with the view that the virus was in some way simpler than other proteins, but whether because it was more primitive than the crystalline, or is a degradation product of them it is of course impossible to say at present. Astbury and Bell (1938) and Astbury (1938 *a, b*) have suggested that the hypothetical virus sub-units of 22  $\text{\AA}$  cube are each linked with one nucleotide of the nucleic acid chain or ring. This fits reasonably with the analytical figures for phosphorus content but as yet there is no direct evidence for the view. The answer to these questions must be found in a far wider study of proteins and nucleoproteins by x-ray methods.

#### 2. *Biological Implications*

So far the discussion has been confined to an elucidation of the modes of aggregation and the internal structure of virus particles, based primarily on an intensive study of tobacco mosaic virus, with its two selected strains, enation and aucuba which are except in the respects mentioned below indis-

<sup>4</sup> More recently the structure of horse serum albumin has been studied by one of us (Fankuchen, 1940) and this seems to offer some analogy to that of tobacco mosaic virus. It is hexagonal,  $a = 96 \text{ \AA}$ ,  $c = 144 \text{ \AA}$  the main reflection being the (0006) at 24  $\text{\AA}$ .

tinguishable. The other viruses, cucumber virus strains, potato virus X strains, and bushy stunt, were studied less intensively and can only be discussed here in a comparative way.

### *Physical Properties*

In physical properties the different virus preparations vary widely (see Bawden and Pirie, 1937 *a* and *b*; 1938 *a*, *b* and *c*). The differences that we observed refer only to the preparations of wet and dry gel. In this respect the behaviour of the three strains of tobacco mosaic virus was indistinguishable, all gave gels of similar texture and birefringence, but it was observable that these were more easily obtained in a well oriented form from the ordinary strain.

Cucumber virus strains gave gels of lower birefringence for wet and for dry gel, and these were definitely not so well oriented. Potato virus X strains gave even worse gels of very low birefringence and poor orientation. No orientation phenomena were observable for bushy stunt virus, and the gels it gave were amorphous.

### *Intramolecular Patterns*

All the strains of tobacco mosaic virus gave indistinguishable intramolecular patterns, as far as the position and intensities of the four principal spots, (0003), (0006), (4482), and (3037), are concerned (see p. 148). Enation and the ordinary strain in solution also gave the same full detailed patterns. The aucuba strain and the cucumber virus were not examined in such detail. We may, however, conclude that they contain the very similar sub-units and particle structure. Potato virus X showed a different intramolecular pattern, (Plate 3, Fig. 21), which, however, was clearly related to that of the tobacco virus. The reflection corresponding to (0006) in tobacco mosaic virus remains as the strongest at 11 Å, but (0003) is replaced by two spots of spacing 33 and 16.5 Å representing the first and second orders of which (0006) is the third. (3140) and (4482) are replaced by arcs of approximately the same spacing, but it is impossible to determine their precise position owing to the poor orientation of the specimens. No difference was detectable between the ordinary and the masked strains of potato virus X. The most obvious interpretation at this stage is that the virus contains similar sub-units, but that these are arranged in a different way to those of tobacco and cucumber virus. In the case of the bushy stunt virus the distinction established between intermolecular and intramolecular patterns breaks down. As the crystals here are true crystals all x-ray reflections must conform to Bragg's law for the crystal unit, which here corresponds to the intermolecular pattern observed for the other viruses. Powder photographs at short distances, however, do show definite concentrations of scattering at about 11 and 4.5 Å, of which no more can be said than that they are typical of proteins in general and not unlike those

given by the other viruses. It may be possible to get more information on this point by examining single crystals, though so far these have proved too small for the purpose.

### *Intermolecular Patterns*

The different varieties of virus showed distinct differences in their intermolecular patterns. These differences are of two kinds, namely, in the spacing of the pattern and in the relative intensities of the different spots. Within the accuracy of measurement of about one-fifth per cent, all strains of tobacco mosaic virus give the same dry gel spacing. It must be emphasized that this reproducibility is one of the most notable characteristics of the virus preparations. Centrifuged sap, dried top and bottom solutions of different concentrations, and dried crystals prepared from different samples and at different times, give identical spacings to this degree of accuracy. Both varieties of cucumber virus give a different spacing though the difference is small, 128 Å against 132 Å for the tobacco mosaic strains.

The patterns given by the bushy stunt virus are necessarily of a very different kind. The virus was prepared in the form of small crystals which on microscopic examination appeared to be regular dodecahedra, which are completely isotropic (Bawden and Pirie, 1938 *b*). This indicates the probability of a spherical rather than an elongated particle. For the x-ray examination powder methods were used, the virus being examined first of all in its mother liquor and subsequently air dried (Bernal, Fankuchen, and Riley, 1938). The results of the measurements are shown in Table I. They correspond to those of a body-centred cubic structure. This is in perfect agreement with the habit of the crystals, for according to Federov's law the rhombohedral habit corresponds to body-centred packing. The size of the cell in the wet case is 394 Å and in the dry case 318 Å. Their ratio corresponds within errors of measurement to a shrinkage observed on the crystals themselves. Using this cell size and the observed density of 1.286 for the wet crystals, the wet particle molecular weight would appear to be 24,000,000. Bawden and Pirie (1938 *b*) estimated the water content of these crystals to be 55 per cent and if this is the case the dry particle molecular weight would be 10,800,000 compared to 8,800,000 the molecular weight measured by McFarlane and Kekwick (1938). More recently, Neurath and Cooper (1940) have reported a molecular weight of 10,600,000, based on sedimentation and diffusion measurements. It has so far proved impossible to measure the density of the dry crystals, and thus to measure the dry molecular weight directly. The value given above would indicate a density of only 1.12 for the dry crystals, which would appear to show that there is considerable zeolitic loss of water. The diameter of the dry particle would appear to be 276 Å or much greater than that of the tobacco mosaic type.

The other viruses did not provide any recognisable intermolecular pattern, though, particularly in the case of potato virus X, exhaustive search was made for such a pattern. This may be due to the larger size of the virus particles or to their irregular shape. Further work is obviously needed here.

The other type of difference between intermolecular patterns of the other virus strains is that of intensity. Plate III, Fig. 18 and Table I show the different reflections of tobacco and cucumber viruses and their estimated intensities. All these were taken from specimens prepared in an identical way and examined in the same orientation. The three strains of the tobacco virus do show definite differences of intensity. In view of the discovery of the secondary orientation of the particles it might seem that the differences are due to this cause, and, indeed, to a certain extent this may be so, the tendency to orientation being possibly more marked in the normal tobacco mosaic strains than in the aucuba and enation strains. This, however, cannot account for the increased intensity of the reflections from the  $(12\bar{3}0)$  planes, which, occurring at intervals of approximately  $30^\circ$ , are comparatively independent of secondary orientation. The cucumber virus shows this difference in a far more marked form, the unresolved doublets of planes  $(22\bar{4}0)$  and  $(31\bar{4}0)$ , which is only visible in tobacco after heavier exposures, are here prominent. The specimens of tobacco mosaic virus prepared in 1938 were found to give a pattern which in intensity was very different from that of the earlier preparations. Further this pattern did not itself remain constant (Table I). What is notable is the increase in intensity of the  $(22\bar{4}0)$  and the variability of  $(12\bar{3}0)$ . It was thought that these changes might be due to ageing and some of the specimens were sent back to Mr. Bawden for infectivity tests but were found to behave quite normally. It is possible that these differences are not due to changes in the particle itself, but merely because of its association with water. The line intensity is in fact very sensitive to the amount of water present. An old specimen of tobacco mosaic virus which when dried *in vacuo* gave an excellent four line pattern with  $(1\bar{1}00)$  most intense and interparticle distance of  $150 \text{ \AA}$ , gave on exposure to air on a damp day an extremely poor pattern with  $(12\bar{3}0)$  strong and  $(1\bar{1}00)$  weaker and interparticle distance of  $153 \text{ \AA}$ . Attempts were made to estimate these differences quantitatively by photometer records of the film densities. Owing to the orientation effect, however, the relative intensities can only be used for comparison purposes.

It is difficult to assess the significance of the differences in the intermolecular patterns, but they are probably due to slight differences in the shape of the cross-section and of its association with water. Their full elucidation requires further work in conjunction with a closer study of their intramolecular patterns. They have some value, however, for comparative purposes.

### *Classification*

The comparison between the intramolecular structure of tobacco, cucumber, and potato viruses points clearly to a common pattern. Already, on the basis of the x-ray analysis alone, it is possible to arrange the specimens we have examined on a scale of similarity and differences which is shown in Table I. The three varieties of tobacco mosaic virus which only show slight differences in intensity in their intermolecular patterns and none at all in their intramolecular, might be counted as varieties in a species. The related species would be that of cucumber mosaic virus, which shows marked differences in the intermolecular pattern, not only in intensity but also in spacing. A different genus would be represented by the potato virus, where the intramolecular pattern indicates a different arrangement of similar sub-units. Still another would be given by the bushy stunt virus, in which although the intramolecular pattern is roughly the same in character, no orientation phenomena can be observed. All these virus specimens together show patterns which have more similarities amongst each other than with other proteins though it should be pointed out that those examined so far by x-rays are a small and unrepresentative sample.

These similarities and differences are seen to run closely parallel to those obtained by Bawden and Pirie (1937 *a* and *b*; 1938 *a* and *b*; 1939) on clinical and serological grounds, and it may be hoped that the x-ray method may prove of value in explaining the structural characters underlying the differences between strains and species of virus and similar bodies.

### *General Conclusions*

The provisional picture that we can now give of plant virus particles has already modified earlier conclusions in several respects. The virus that has been most thoroughly studied, that of the tobacco mosaic disease, seems to have very elongated particles of variable length, which we now recognise are in part artefacts. Virus particles in the plant are definitely shorter and possibly all of the same length. In bushy stunt virus, which gives cubic crystals, they must be approximately spherical. The anisotropic character of the other viruses, so interesting from the colloid chemical standpoint and so useful for their x-ray examination, seems now to have been somewhat of a happy accident, brought about by the tendency of cylindrical molecules to aggregate along their length. It must be emphasized, however, that if this is the case, all trace of the length of the original particles may have been lost in the resulting elongated particle.

The x-ray evidence points to a virus particle of complex structure. It is more analogous to a protein crystal than a protein molecule. Its internal regularity is very considerable. The crystallinity of the virus goes further than the aggregation of similar particles that produce the visible crystals or liquid crystals, it is a property of the isolated particles of the virus.

The arrangement seems to be one of small sub-units,  $11 \times 11 \times 11 \text{ \AA}$  definitely of a smaller order than those of crystalline protein molecules. There are indications that the nucleoprotein of the particle has a different internal structure from that of normal crystalline proteins and that it is in some ways simpler.

### *Biological Significance*

This is not the place to discuss the biological significance of these observations. Until more is known of the structure and biochemical functions of systems such as bacteria, whose chemical activities are usually taken as criteria of life, the crystalline nature of the particles that we have studied cannot in itself be given a biological significance nor give an answer to the question as to whether they are or are not the infective agent (Bawden and Pirie, 1937 *a*), nor to the far more metaphysical question as to whether they are to be considered living organisms (Pirie, 1937). We would moreover like to emphasize that the only fully reliable facts that are here established are the physical and x-ray measurements. The hypotheses used to explain them are more or less provisional and it would be premature to base elaborate or detailed interpretations of structure and function on the evidence available at this stage of the work.

The chief utility of this work up to the present has probably lain in the extension to the biological field of two new methods, the technique of the study of oriented aggregates and the x-ray examination of complex structures of very long spacings. We have shown that this latter technique is by no means difficult and we may therefore hope that many other such structures will be discovered by its means. Effectively by the use of ordinary x-rays at small angles we can measure any regularities of the order of  $1000 \text{ \AA}$  or  $1/10 \mu$  and this is close to the lower resolvable limit of the ultraviolet microscope and has already been reached by the electron microscope. It should further be possible to push the examination of fine structures from that obtainable by the microscope right down to atomic dimensions.

The study of the virus preparations by these methods has brought to light hitherto unsuspected or at least undemonstrated properties of colloidal aggregates. It has been demonstrated that linear colloidal particles are arranged, even in dilute solution, in aggregates as regular as those of a crystal. The distance between them can be varied with the ionic character of the medium and this proves the existence of long range interparticle forces and indeed provides quantitative confirmation of the theories of these forces. Further by combining the x-ray studies with optical observations it has been possible to build up a theory of gel formation and thixotropy that may prove of value. The viruses of the tobacco mosaic type owing to their extreme uniformity and stability are indeed an ideal material for the controlled studies of colloids. Finally,



it must be emphasized that with such a bewildering set of new phenomena, the work here recorded represents only a preliminary and rough survey and that many more man-years of work will be required before exact and reliable interpretations can be expected.

We are greatly indebted to Mr. N. W. Pirie and Mr. F. C. Bawden for supplying us with the virus preparations used in this work and to Mr. C. Chapman for the construction of the apparatus. Miss F. Bell took some of the earliest intramolecular photographs of the virus preparations. Dr. Crowfoot was of the greatest assistance during the final revision of the paper and was responsible for the preparation of many of the illustrations. A grant from the Royal Society made possible the purchase of the x-ray apparatus at Birkbeck College, and one of us (I. Fankuchen) has been assisted by a grant from the Department of Scientific and Industrial Research.

#### SUMMARY

These papers give an account of an optical and x-ray examination of preparations of plant virus substances isolated by Bawden and Pirie, in particular of those of tobacco mosaic disease. They open with a historical survey of the work, indicating the order in which new phenomena were discovered. The subsequent treatment is divided into three parts:

I. Introduction and preparation of specimens.

II. Modes of aggregation of virus particles.

III. (1) The structure of the particles.

(2) Biological implications.

Part I, after an historical introduction, describes the method of preparation, from solutions of the virus, of optically oriented specimens of different concentrations. For their examination special x-ray apparatus was developed, in particular cameras working with very low angles and capable of indicating spacings up to 1000 Å.

Part II is concerned with the phenomena of aggregation of the virus particles in solutions. Two modes of aggregation are observed, a disoriented sol at low concentrations and an oriented sol passing continuously over to an oriented gel at higher concentrations. Solutions of medium strength divide into two layers on standing. The top layer, which is more dilute, *i.e.* of concentrations below 1.6 to 4 per cent is an isotropic liquid which shows flow birefringence and other phenomena indicating the presence of long thin particles. The bottom layer in equilibrium with it, and also the higher concentrates prepared by centrifugation, are spontaneously doubly refractive and consist of regions in which the particles are parallel. X-ray investigation shows that the particles are also equidistant and that the sol is a new kind of liquid crystal with a regular hexagonal arrangement in cross-section. The distances between the particles depend simply on the concentration, indicating the homogeneity of their dis-

tribution. The equilibria between top—and bottom—layer solutions and the special forms which the latter can exhibit—tactoids and reversed spirals—are also discussed. Preparations with concentrations of 30 per cent and over have gel-like properties and become stiffer as the water content decreases, but there are no abrupt transitions. At pH 7 in the absence of salt these gels swell until a concentration in equilibrium with the top layer is reached, that is down to concentrations of 4 per cent–1.6 per cent. Nearer the isoelectric point, at pH 3.4, or in ammonium sulfate solution, the degree of swelling is much more limited and an equilibrium is reached in which the distance between the particles depends on the pH or the salt concentration. The minimum interparticle distance at the isoelectric point or for high salt concentrations is about 175 Å. The original “crystals” observed by Stanley are shown to be gels of this type in the form of small elongated tactoids. On drying, further shrinkage takes place and the resulting air dry gel has an interparticle distance of only 152 Å. In these gels the arrangement of particles in the cross-section is so perfect that each specimen is a two-dimensional single crystal. The conclusion of this study is that the virus preparations consist of approximately cylindrical particles about 150 Å in diameter. Their length cannot easily be estimated by x-ray methods, but the minimum value of 1,500 Å found by Kausche, Pfankuch, and Ruska (1939) is compatible with the x-ray evidence. Much longer particles formed by aggregation of these also occur. The forces maintaining the particles equidistant and parallel in these gels are probably due to the ionic atmospheres surrounding them. It is shown that for long particles the mutual energy due to the ionic atmospheres is considerably greater than their temperature motion at ordinary temperatures, and consequently they must arrange themselves parallel and in a regular two-dimensional lattice. Further consideration of these phenomena leads to an explanation for the other colloid phenomena shown by the viruses, particularly the formation of tactoids and gels. The spindle-shaped form of the tactoids is shown to be due to anisotropic surface tension due to the parallel arrangement of the long particles on the surface. Dilute gels appear to be composed of tactoids of a multiply branched character showing thixotropic behaviour. This theory may have wide extensions to other colloid systems.

In Part III, Section 1 deals with the x-ray evidence on the internal structure of the particles. Even in solution, they have an inner regularity like that of a crystal. Virus preparations are thus in a sense doubly crystalline. Closer analysis reveals that the x-ray patterns are not directly comparable to those of a crystal as many of the reflections do not obey Bragg's law, but can be understood on the theory of gratings of limited size. The structure seems to consist of sub-units of the dimensions of approximately 11 Å cube, fitted together in a hexagonal or pseudohexagonal lattice of dimensions— $a = 87$  Å,  $c = 68$  Å. Contrary to what earlier observations seemed to indicate, the particle seems

to be virtually unchanged by drying and must therefore contain little water. There are marked resemblances with the structure of both crystalline and fibrous protein, but the virus structure does not belong to any of the classes hitherto studied. There are indications that the inner structure is of a simpler character than that of the molecules of crystalline proteins.

Part III, Section 2 contains a comparative study of the optical and x-ray examinations of three strains of tobacco mosaic virus, two of cucumber disease virus, two of potato virus X, and the virus of bushy stunt disease of tomato. In the last case x-ray measurement confirmed the deduction from its cubic crystal habit that it was composed of spherical rather than long particles, and showed that these had a diameter when dry of 276 Å and were arranged in a body-centred cubic close packing. This single example is sufficient to show that the elongated particle form which gives rise to all the anomalous physical properties of the other viruses studied is of no essential biological importance. The similarity and differences observed between the physical properties of these preparations run closely parallel to their clinical and serological classification. Finally, the biological implications of these results are discussed together with possible applications of the new methods of examination to the study of colloid and biological problems.

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# DETERMINATION OF CERTAIN AMINO ACIDS IN CHYMOTRYPSINOGEN, AND ITS MOLECULAR WEIGHT

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Chymotrypsinogen is a crystalline protein, which has been isolated from acid extracts of fresh cattle pancreas by Kunitz and Northrop (1). This protein is transformed by minute amounts of trypsin into an active proteolytic enzyme, originally called chymotrypsin (1), but later designated  $\alpha$ -chymotrypsin by Kunitz (2). This paper deals with the determination of the sulfur amino acids and of tyrosine and tryptophane in chymotrypsinogen, kindly put at our disposal by Dr. Kunitz.

Semi-micro methods for the accurate determination (in protein hydrolysates) of the sulfur amino acids, cystine, cysteine, and methionine, and of sulfate sulfur have been described in recent publications (3-6) from this laboratory. A system of analysis has been developed in which each of these constituents is determined by at least two independent methods. Hydrolysis is carried out in an inert atmosphere with HI in presence of hypophosphite, with HCl or with HCl in the presence of urea (7).

During HI digestion methionine ( $\text{CH}_3\text{—S—CH}_2\text{·CH}_2\text{·CH(NH}_2\text{)·COOH}$ ) is split into methyl iodide and the S-lactone of homocysteine ( $\text{HS·CH}_2\text{·CH}_2\text{·CH(NH}_2\text{)·COOH}$ ). Both split products are determined, the former as volatile iodide, the latter iodometrically (5). HI hydrolysis leaves cysteine unchanged, while cystine is reduced to cysteine, so that the sum of these compounds can be determined as cysteine by iodometric oxidation (5). Cysteine and cystine are determined separately by our photometric method in HCl hydrolysates (3, 4).

Acid-insoluble humin in HCl hydrolysates interferes with the cysteine determination, since the humin precipitate may contain appreciable amounts of cysteine, as shown first by Lugg (8). With certain proteins (e.g. egg albumin and lactalbumin) the formation of acid-insoluble humin can be almost entirely prevented by carrying out the HCl hydrolysis in the presence of urea (7); under these conditions satisfactory results for both cysteine and cystine are obtained.

The Sullivan method (*cf.* 3, 6), which is specific for cystine, cannot be inter-

puted if a hydrolysate contains both cystine and cysteine, since the latter develops more color than the former. This method is used as a check on the cystine values (*cf.* 3, 6). Sulfate S is determined as  $H_2S$  in HI hydrolysates (5) and also by a micro gravimetric procedure as  $BaSO_4$  in a separate HCl hydrolysate (5). Total sulfur in the protein is determined by an adaptation (6, 9) of the Pregl method.

TABLE I  
*Methionine, Cystine, and Sulfate in Chymotrypsinogen*

Substance (1)	Hydrolysis (method) (2)	Methionine determined as		Cystine determined as			Sulfate sulfur determined as	
		Volatile iodide (3)	Homo-cysteine (4)	Cys-teine* (5)	Cystine (6)	Cysteine + cystine (7)	$H_2S$ (8)	$BaSO_4$ (9)
mg.		per cent	per cent	per cent	per cent	per cent	per cent	per cent
237.3	HI (a)	1.19	1.14	4.49	0	4.49	0.62	
253.0	" (b)	1.22	1.23	4.57	0	4.57	0.59	
188.3	HCl (c)			1.11	3.34	4.45		
91.4	" (d)			1.26	3.23	4.49		
101.54	" (e)							0.64
Total used for analysis 872		Average 1.20				Average 4.50	Average 0.62	
Corrected†		1.22 (0.262 as S)				4.59 (1.225 as S)		

\* Cysteine is calculated as half-cystine (MW = 120).

† For sulfate content = 1.94 per cent.

(a) (b) 6 hrs. at 150°, method (5).

(c) 8 hrs. at 130°, method (3).

(d) 8 hrs. at 130°, in the presence of urea, method (3, 7).

(e) 6 hrs. at 130°, method (5).

For casein, lactalbumin, reduced lactalbumin (6), crystalline egg albumin (6), blood fibrin (7), and thyroglobulin (10), we have shown that the sum of the average values for methionine, cystine, cysteine, and sulfate sulfur accounts within 1 per cent for the total sulfur determined by elementary analysis. This concordance establishes the significance of the values obtained for the individual sulfur amino acids.

From Table I it can be seen that chymotrypsinogen contained some sulfate (0.62 per cent sulfate S or 1.94 per cent calculated as  $SO_4$ ) which had not been removed by dialysis. The methionine content is 1.22 and that of cystine + cysteine 4.59 per cent;<sup>1</sup> the agreement between values obtained by independent methods is excellent. In HCl hydrolysates about one quarter of the total cystine is present as cysteine.

<sup>1</sup> Cysteine (MW = 121) is calculated as half-cystine (MW = 120).

In Table II it is shown that methionine S and cystine + cysteine S account accurately for total sulfur less sulfate sulfur. Chymotrypsinogen contains 1.48<sub>4</sub> per cent of protein sulfur.

TABLE II  
*Sulfur Distribution and Molecular Weight of Chymotrypsinogen*

Constituent	Sulfur	Per cent of protein sulfur	Constituent	Sulfur
	<i>per cent</i>			<i>per cent</i>
Methionine.....	0.262	18	Total S (Pregl).....	2.07
Cystine + cysteine.....	1.225	82	Sulfate S.....	0.62
Protein S.....	1.487		Protein S.....	1.45
			(Corrected)*.....	1.48

↑————— Av. Protein S = 1.48<sub>4</sub> per cent —————↑

$$\frac{\text{Methionine S}}{(\text{Cystine} + \text{cysteine}) \text{ S}} = \frac{N_{\text{methionine}}}{N_{\text{half-cystine}} + N_{\text{cysteine}}}$$

$$\frac{0.262}{1.225} = \frac{1}{4.67} = \frac{2}{9.35} = \frac{3}{14.03} = \frac{4}{18.7}$$

Methionine: half-cystine + cysteine = (a) 3:14 or (b) 4:19

$$\frac{\text{Per cent cysteine}}{\text{Per cent cystine}} = \frac{N_{\text{cysteine}}}{N_{\text{half-cystine}}}$$

$$\frac{1.29}{3.30} = \frac{1}{2.56} = \frac{2}{5.12} = \frac{3}{7.67} = \frac{4}{10.2} = \frac{5}{12.8} = \frac{6}{15.4}$$

Cysteine: half-cystine = 4:10

Methionine : half-cystine : cysteine : sulfur  
3 : 10 : 4 : 17

Minimum molecular weight = 36,600

Molecular weight by osmotic pressure = 36,000  
(Kunitz and Northrop (1))

\* Corrected for SO<sub>4</sub> content = 1.94 per cent.

From the distribution of the sulfur amino acids the minimum molecular weight of chymotrypsinogen can be calculated, on the basis that the material analyzed is a pure chemical individual and that a protein molecule must contain integral numbers of the constituent amino acids. The molecular weight of a protein is given by equation (1):

$$M_p = \frac{M_i \times 100 \times N_i}{(\text{Per cent})_i} \quad (1)$$



$M_p$  and  $M_i$  represent respectively the molecular weight of the protein and of an individual amino acid,  $N_i$  is an integer and represents the number of residues of an individual amino acid, (per cent) $_i$  is the content, in per cent by weight, of the individual amino acid in the dry protein. Cystine (CyS—SCy, MW = 240) is the equivalent of two amino acids, it has, therefore, to be considered in terms of half-cystine residues ([CyS—], MW = 120). By definition, then, the number of half-cystine residues ( $N_{\text{half-cystine}}$ ) in a protein must always be an *even integer*, while the number of cysteine residues ( $N_{\text{cysteine}}$ ) may be an even or an odd whole number.

If a single amino acid ( $a$ ) has been determined, the minimum molecular weight is obtained from equation (1) by taking  $N_a = 1$ . If two amino acids ( $a$ ) and ( $b$ ) have been determined, minimum values for  $N_a$  and  $N_b$  are obtained by setting up two simultaneous equations for  $M_p$ , resulting in equation (2):

$$\frac{(\text{Per cent})_a}{(\text{Per cent})_b} \times \frac{M_b}{M_a} = \frac{N_a}{N_b} \quad (2)$$

in which the left side is preferably expressed as a *proper fraction*; i.e., one in which the numerator is smaller than the denominator. The conversion of this proper fraction into the *smallest simple fraction* (i.e. one in which both numerator and denominator are integers) yields the number of residues, of ( $a$ ) and ( $b$ ) per minimum molecular weight. For the sulfur amino acids equation (2) can be simplified as given in equations (3) and (4), since in these cases<sup>1</sup>

$$\frac{M_b}{M_a} = 1.$$

$$\frac{\text{Per cent methionine sulfur}}{\text{Per cent (cystine + cysteine) sulfur}} = \frac{N_{\text{methionine}}}{N_{\text{half-cystine}} + N_{\text{cysteine}}} \quad (3)$$

$$\frac{\text{Per cent cysteine}}{\text{Per cent cystine}} = \frac{N_{\text{cysteine}}}{N_{\text{half-cystine}}} \quad (4)$$

Since the protein sulfur of chymotrypsinogen has been accurately determined (with an error of less than 1 per cent) and has been fully accounted for as cystine, cysteine, and methionine, (each of which contains nitrogen and sulfur in equiatomic proportions) the relationship given in equation (5) obtains:

$$N_{\text{sulfur}} = N_{\text{methionine}} + N_{\text{half-cystine}} + N_{\text{cysteine}} \quad (5)$$

In Table II the number of S atoms and the number of residues of the sulfur amino acids in the minimum molecular weight of chymotrypsinogen are calculated from equations (1) to (5) using the experimental data reported. The figures for methionine S and for (cystine + cysteine) S are expressed as a *proper fraction* and converted into a *simple fraction*. It can be seen that for

1 and 2 methionine residues, the corresponding values for half-cystine + cysteine residues differ by 7 and 4 per cent, respectively, from the nearest integers; these differences are outside of the error of the methods. However, for 3 and 4 methionine residues per mol the corresponding half-cystine + cysteine values differ only by 0.4 and 1.6 per cent from the integers 14 and 19, respectively. These values for  $N$ , together with the percentages found, lead to a minimum molecular weight for chymotrypsinogen of either (a) 36,600 with 17 atoms of sulfur and 3 and 14 residues, respectively, of methionine and of half-cystine + cysteine or (b) 49,400 with 23 atoms of sulfur and 4 and 19

TABLE III  
*Tyrosine and Tryptophane in Chymotrypsinogen*

Sample	Tyrosine		Tryptophane	
	Hydrolysis with		Hydrolysis with	
	NaOH	NaOH—SnCl <sub>2</sub>	NaOH	NaOH—SnCl <sub>2</sub>
mg.	per cent	per cent	per cent	per cent
20.9	2.92		5.09	
21.5		2.97		5.29
30.0	2.99		4.88	
30.9		2.84		5.36
38.4	2.82		4.96	
39.8		2.84		5.54
Total used for analysis 181	Average 2.90		[Average 4.98]	Average 5.40
Corrected*	2.96			5.51

\* For sulfate content = 1.94 per cent.

residues, respectively, of methionine and half-cystine + cysteine. The decision between (a) and (b) can under favorable conditions be made on the basis of equation (4). The per cent of cysteine is divided by that found for cystine and converted into a series of *simple* fractions (Table II). Within the experimental error, the smallest possible<sup>2</sup> set of figures, is 4/10, which is in agreement with (a). This value (36,600) is the actual molecular weight of chymotrypsinogen, since Kunitz and Northrop (1) have found by osmotic pressure measurements a molecular weight of 36,000.

We have recently (11) described a micro method for the photometric determination of tryptophane, tyrosine, diiodo-tyrosine, and thyroxine in protein hydrolysates (based on procedures developed by Lugg (12) from the Folin-Ciocalteu (13) method). In the course of the determination tyrosine is quanti-

<sup>2</sup> The number of half-cystine residues should be an even integer, this eliminates 2/5 from consideration.

tatively separated from tryptophane, which is isolated as an insoluble mercury compound. The Millon reaction is used for the photometric determination of both amino acids; correction factors were established to compensate for destruction during hydrolysis. Hydrolysis is carried out in sealed tubes with both NaOH and NaOH—SnCl<sub>2</sub>. Tyrosine destruction is about 1.5 per cent greater in NaOH than in NaOH—SnCl<sub>2</sub> hydrolysates. Using such a correction the same results for the tyrosine content of egg albumin, fibrin, and lactalbumin are obtained with both methods of hydrolysis (11). It can be seen from Table III that this holds also for the tyrosine in chymotrypsinogen. The tyrosine content of chymotrypsinogen is 2.96 per cent (Table III), that is 6 tyrosine residues per mol (Table IV).

TABLE IV  
*Composition and Molecular Weight of Chymotrypsinogen*

Constituent (1)	Per cent (2)	No. of residues (3)	Molecular weight (4)	Percentages calculated for MW = 36,700 (5)	Deviation (5) - (2)
Methionine.....	1.22	3	36,600	1.22	0.00
Half-cystine (SH + SS) .	4.59	14	36,600	4.58	+0.01
Protein sulfur.....	1.48 <sub>4</sub>	17	36,700	1.48 <sub>4</sub>	0.00
Tyrosine.....	2.96	6	36,700	2.96	0.00
Tryptophane.....	5.51	10	37,000	5.56	-0.05
			Average 36,700		
Cysteine .....	1.29	4		1.31	-0.02
Half-cystine [CyS-].....	3.30	10		3.27	+0.03

In the case of tryptophane the destruction of the pure amino acid by NaOH (*cf.* 11) is much greater (7 per cent) than by NaOH—SnCl<sub>2</sub> (2 per cent). However, in proteins such as egg albumin, fibrin, globin, and thyroglobulin, we find that the tryptophane is more stable towards alkali, so that the same results are obtained both after alkaline and alkaline-stannite hydrolysis. From Table III it can be seen that chymotrypsinogen differs from these proteins, being about 9 per cent lower in tryptophane by alkaline hydrolysis without stannite. The results with alkaline-stannite hydrolysis indicate a tryptophane content of 5.51 per cent or 10 residues per mol (Table IV). Chymotrypsinogen has the highest tryptophane content of any protein so far on record.<sup>3</sup>

The experimental data are summarized in Table IV. The molecular weights calculated from the determinations of the individual constituents (according to equation (1)) are given in column (4). The molecular weight of chymo-

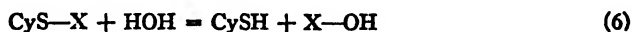
<sup>3</sup> This high tryptophane content had already been found by Kunitz (personal communication). Our unpublished experiments indicate that the tryptophane content of beta and gamma chymotrypsin is still higher.

trypsinogen obtained from these determinations is 36,700. The deviations from the mean are 100 (or 0.3 per cent) for the sulfur amino acids and 300 (or 0.9 per cent) for tryptophane. A detailed discussion of the data and their implications regarding the structure of the chymotrypsinogen molecule will be postponed until similar data have been obtained for alpha, beta, and gamma chymotrypsin.

Chymotrypsinogen has no reactive SH groups, since we find that the nitroprusside test is negative in the presence of ammonia. Nevertheless hydrolysis with HCl yields 4 cysteine groups per mol. The appearance of these Cy—SH groups on hydrolysis may be due to one of the following possibilities:

(a) The Cy—SH groups are actually present in the native protein, but unreactive (this would also include hydrogen bonds and salt bridges).

(b) They are not present in the native protein, the sulfhydryl hydrogen of the cysteine being substituted for by X. On HCl hydrolysis Cy—SX groups yield cysteine according to equation (6):



The question whether there are Cy—SH or Cy—SX groups present in native chymotrypsinogen remains unsolved.<sup>4</sup> It is hoped that experiments on denatured chymotrypsinogen with porphyrindin along the lines developed by us for egg albumin (14) or by the technique of Anson (15, 16) will shed light on this question.

It is generally recognized that some uncertainty is attached to all amino acid determinations, since they are carried out in hydrolysates. The study of the behavior of the free amino acids under varying conditions of hydrolysis (*cf.* 5, 11) and the establishment of correction factors overcomes this difficulty to some extent. There always remains the possibility that in a specific protein structure an amino acid is unusually labile and subject to increased hydrolytic destruction and other reactions. Examples are the sensitivity of the cystine in the insulin molecule towards HI hydrolysis, whereas it is quite stable during HCl hydrolysis (17); also the greater destruction of tryptophane in chymotrypsinogen during alkaline hydrolysis as compared to alkaline stannite hydrolysis (*cf.* Table III). However, in the case of the sulfur amino acids and of the iodoamino acids the analysis of hydrolysates may yield unequivocal evidence. If—as in chymotrypsinogen—the protein sulfur, determined by elementary analysis, is fully accounted for by methionine and cystine + cysteine in hydrolysates, then we have conclusive evidence that the amount of these amino acids actually present in the protein has been determined.

<sup>4</sup> It should be recognized that the appearance not only of Cy—SH groups but of SH groups in general may be due to (a) activation of free SH groups, (b) hydrolysis of S—X groups (equation 6), and (c) hydrolysis of S—S groups ( $\text{S—S} + \text{HOH} = \text{SH} + \text{S—OH}$ ). This will be discussed elsewhere.

As is apparent from its method of preparation and from some of its properties, the crystalline chymotrypsinogen of Kunitz and Northrop is a protein of exceptional homogeneity, purity, and stability. The excellent agreement of the data in Table IV is primarily due to this fact. On the other hand these results establish confidence in the accuracy and precision of the analytical methods developed by us. The results of recent physical and physicochemical studies have clearly demonstrated the fact that native proteins are compounds with well defined molecular mass and shape. The earlier chemical investigations are not very conclusive in this respect, partly because the proteins analyzed were impure, partly because most methods of amino acid analysis lack sufficient accuracy and precision. The results in Table IV supplement the physical and physicochemical data and show that chymotrypsinogen is a well defined chemical entity, obeying the law of multiple proportions.

### Methods

The sample of chymotrypsinogen as obtained from Dr. Kunitz, had been 7 times recrystallized, dialyzed, and then dried *in vacuo* and  $\text{CO}_2$  snow. It was equilibrated in air for 2 days; the change in weight was insignificant (0.1 per cent). The air-dried material was used for all analytical procedures, the weights being corrected for moisture content.

**Moisture Determination.**—53.185 mg. were dried *in vacuo* over  $\text{P}_2\text{O}_5$  at  $100^\circ\text{C}$ . to constant weight (4 hours); loss 3.544 mg. = 6.66 per cent of moisture. A second sample of 57.144 mg. lost 3.698 mg. = 6.47 per cent. Average moisture content = 6.57 per cent.

**Determination of Total Sulfur.**—We are indebted to Mr. Wm. Saschek for these determinations, carried out by an adaptation of the Pregl method (6, 9). The result reported in Table II (2.07 per cent) is the average of two determinations, 27.08 mg. and 27.09 mg. of chymotrypsinogen yielded 4.04 mg. and 4.09 mg. of  $\text{BaSO}_4$ , respectively, = 2.05 and 2.09 per cent of sulfur, respectively. There was no ash.

**Micro Gravimetric Determination of Sulfate S.**—This determination was carried out as described previously (5). 101.54 mg. of chymotrypsinogen were hydrolyzed under  $\text{CO}_2$  with 5 cc. of 6 N HCl for 6 hours in an oil bath at  $130^\circ\text{C}$ . 4.710 mg. of  $\text{BaSO}_4$  were obtained, sulfate S = 0.64 per cent (*cf.* Table I).

A test with Nessler's reagent for free ammonia was practically negative, indicating that the sulfate was probably combined with basic groups and not present as ammonium sulfate. The average content of sulfate S (0.62 per cent, *cf.* Table I) corresponds to 1.94 per cent of  $\text{SO}_4$ . The average values of the individual determinations were corrected accordingly (*cf.* Table I-IV).

**Determination of Total Nitrogen.**—We are indebted to Mr. Wm. Saschek for these determinations by the micro-Dumas (Pregl) method. 3.639 mg. and 3.192 mg. gave 15.83 and 15.90 per cent of nitrogen respectively.

The nitrogen content of our sample of chymotrypsinogen, corrected for sulfate (1.94 per cent) is 16.18 per cent.

Kunitz and Northrop (1) reported 15.8 per cent of nitrogen for chymotrypsinogen. Their preparation contained about 0.44 per cent of sulfate S equal to 1.37 per cent of sulfate (calculated on the basis of the protein sulfur in Table II), since they find a total sulfur of 1.9 per cent. Corrected for sulfate, the total nitrogen is 16.03 per cent.

The values 16.18 and 16.03 per cent for the nitrogen content of chymotrypsinogen are in reasonable agreement. The establishment of a more accurate value seems highly desirable and is of considerable importance for the interpretation of the conversion of chymotrypsinogen into alpha, beta, and gamma chymotrypsin (2).

*HI Hydrolysis.*—The determinations of methionine, of cystine + cysteine, and of sulfate S were carried out as described previously (5) and are reported in Table I ((a) and (b)). After removal of the HI, the digest had a yellow color. Methyl mercaptan was determined in the  $\text{HgCl}_2$  absorber; no unusual amounts were found (cf. 5). This indicates that the decomposition of the methionine in chymotrypsinogen by HI is similar to that observed with methionine and with other proteins (5). Formation of methyl mercaptan from methyl iodide and  $\text{H}_2\text{S}$  (cf. 5) did not take place to any appreciable extent, since the values for sulfate S, determined iodometrically (as  $\text{H}_2\text{S}$ ) and gravimetrically, were the same within the limits of error (cf. 5).

*HCl Hydrolysis.*—188.3 mg. were hydrolyzed for 8 hours in a bath of  $130^\circ\text{C}$ . with 8 cc. of 6 N HCl in a current of  $\text{CO}_2$ . The hydrolysate was brown and contained a very small amount of solid, acid-insoluble humin. In half of this hydrolysate cystine and cysteine were determined separately by the photometric method (3). 1.11 per cent of cysteine and 3.34 per cent of cystine were found (cf. Table I (c)). The other half was used for a cystine determination by the Sullivan method (cf. 3, 6); we found 5.05 per cent of "cystine." As pointed out in previous publications (3, 6) the Sullivan method does not differentiate between cysteine and cystine and gives high results if a hydrolysate contains both cysteine and cystine. We have repeatedly found (3, 6) that cysteine gives about 50 per cent more color than cystine. Therefore we would expect on the basis of our photometric determination to find about 5 per cent of "cystine" by the Sullivan method. This checks the value obtained above by the Sullivan method (5.05 per cent) and is additional evidence that the difference between protein S and methionine S is accounted for by cystine and cysteine and not by any other sulfur-containing compound.

For the hydrolysis with HCl in the presence of urea 91.4 mg. of chymotrypsinogen were hydrolyzed in a current of  $\text{CO}_2$  for 8 hours in a bath of  $130^\circ\text{C}$ . with 5 cc. of water + 2 gm. of pure urea + 11 cc. of concentrated HCl. After 8 hours the hydrolysate had a slight, pale yellowish color, but had remained perfectly clear. The results are reported in Table I (d). This method of hydrolysis should be generally useful to avoid or minimize humin formation.

*Determination of Tyrosine and Tryptophane.*—This method was described previously (11). The protein was so bulky that in the tubes used for hydrolysis somewhat more than 1 cc. of air had to be sealed in. The  $\text{NaOH-SnCl}_2$  hydrolysates were colorless, while the  $\text{NaOH}$  hydrolysates were distinctly yellow. The extinction coefficient of the yellow color in  $\text{NaOH}$  hydrolysates was determined as described (11). The correction was uniform for different hydrolysates and amounted to 0.04 per cent of tryptophane. The results are given in Table III.

## SUMMARY

1. A preparation of chymotrypsinogen, obtained from Dr. M. Kunitz, was analyzed for sulfur, the sulfur amino acids, tyrosine, and tryptophane.

2. The protein sulfur of chymotrypsinogen was accounted for as methionine, cysteine, and cystine.

3. A method is presented for calculating the minimum molecular weight of a protein from the distribution of the sulfur amino acids. In the case of chymotrypsinogen, the calculated minimum molecular weight was found to be the actual molecular weight.

4. The molecular weight of chymotrypsinogen is 36,700 by amino acid analysis as compared to 36,000 by osmotic pressure measurements of Kunitz and Northrop. Chymotrypsinogen contains per mol 17 atoms of sulfur, 3 residues of methionine, 4 of cysteine, 10 of half-cystine (*i.e.* 5 S—S linkages), 6 of tyrosine, and 10 of tryptophane.

5. The tryptophane content of chymotrypsinogen (5.51 per cent) is the highest of any protein so far on record.

6. Chymotrypsinogen contains no reactive SH groups, although it yields cysteine on hydrolysis. This may be due either to preformed but unreactive SH groups or to S—X groups. The term S—X group is used to denote the substitution of the sulphydryl hydrogen by a constituent X; hydrolysis yields SH groups:  $\text{S—X} + \text{HOH} = \text{SH} + \text{X—OH}$ .

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# THE ACTIVITY OF PERIPHERALLY STORED NEUROHUMORS IN CATFISHES

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## 1. INTRODUCTION

Pouchet (1876) in his studies of the color changes in turbot pointed out that when these fishes were made to change color repeatedly they did so in shorter and shorter intervals of time as though they were perfecting a habit. Similar conditions were noted later by van Rynberk (1906) in *Rhomboidichthys* and *Solea*, by Babák (1910) in the larvae of salamanders, by Sumner (1911) in *Lophopssetta*, by Mast (1916) in *Paralichthys*, by Sumner and Wells (1933) in *Lebistes*, by Parker (1934) in *Ameiurus*, and by Matsushita (1938) in *Parasilurus*. Mast pointed out the possible relation of the graded shortening of the times of response to the process of learning. A similar gradual shortening of the color reaction times in fishes has been recently recorded by Parker and Brower (1937) who suggested that in *Fundulus* the initial slowness of the first few color changes in a given series might well result from a residue of the opposing neurohumor left over from a previous lengthy response and that such slowness might continue to be noticeable till the last trace of this residue had disappeared. Such an interpretation of slow initial chromatic responses is open to experimental tests and it is one of the objects of this paper to discuss the outcome of such tests.

The work herein reported was carried out on the common eastern catfish, *Ameiurus nebulosus*. It was suggested by recent studies in which it was shown that the chief and perhaps exclusive agents in the color changes of *Ameiurus* are three: adrenalin, acetylcholine, and intermedin. Of these adrenalin is the sole blanching agent and appears to come from the concentrating nerve fibers of this fish. The other two are darkening agents, acetylcholine from the dispersing nerve fibers, and intermedin from the pituitary gland (Chang, Hsieh, and Lu, 1939; Parker, 1940). Intermedin is carried from its region of origin by the blood and lymph to the melanophores of the skin where it acts on these color cells directly. Adrenalin and acetylcholine appear to originate in close proximity to the melanophores which they activate and from their respective sets of chromatic nerve terminals. They too act directly on the melanophores (Parker, 1941).



Intermedin is freely soluble in water and is carried throughout a given animal in its blood and lymph. In consequence the action of this agent is general and rapid and may take place in a catfish in a few minutes. This and other like neurohumors have been designated in general as hydrohumors (Parker, 1935). Caudal bands in catfishes blanch or darken only very slowly. According to Abramowitz (1936) a single dark caudal band composed of only one ray will blanch in a pale fish in some 3 days. One composed of three rays becomes pale under like circumstances in about 10 days. So slow a process as this cannot depend upon aqueous solution solely. It has been suggested that the neurohumor concerned with blanching, adrenalin, spreads by diffusion not through the aqueous constituents of the tissues but through their fatty or lipoid components (Parker, 1933). The same is true of the nervous darkening agent, acetylcholine. Because of their relation to fatty materials these two neurohumors and others like them have been called lipohumors. Both adrenalin and acetylcholine, however, are also soluble in water. Hence they may under other circumstance act as hydrohumors. Experimental tests show that as hydrohumors, *i.e.* when these substances are dissolved in water, they quickly excite the whole melanophore system, but as lipohumors and in oil they are locally restricted and relatively slow in action. The responses of melanophores to mixtures of adrenalin or of acetylcholine in oil are of first importance in the present research.

## 2. Adrenalin

The adrenalin used in these experiments was the whitish powder manufactured by Parke, Davis, and Company and dispensed by them as such. For the purposes of these tests this powder was further triturated in a mortar, and then mixed with oil to a known dilution. Peanut oil, sesame oil, and Italian olive oil were tried. Olive oil because of its fluidity and inertness toward the fishes was finally used exclusively. Known weights of finely pulverized adrenalin were ground further with definite volumes of olive oil and agitated for a day or so. The strongest mixture was 0.1 gm. of adrenalin in 10 cc. of oil which was conveniently designated as one part in a hundred. From this mixture further dilutions were made to the weakest, one part of adrenalin in 100,000,000 parts of oil. All mixtures from 1:100 to 1:10,000 inclusive showed cloudiness. After these mixtures had been standing a day or so there was a varying amount of sediment on the bottom of their glass containers. Mixtures from 1:100,000 to 1:100,000,000 were indistinguishable in clearness from pure olive oil. The mixture 1:100,000 after filtration through good filter paper and after having been decanted was found still to be active in concentrating melanophore pigment. It is therefore probable that adrenalin is more or less soluble in commercial olive oil. The stronger concentrations of adrenalin injected into the fishes were in all probabilities saturated solutions of this agent in oil with a certain amount of the neurohumor in suspension.

When 0.1 cc. of a mixture of adrenalin one part in a hundred of olive oil is injected subcutaneously into the flank of a dark catfish, a pale spot will appear

over the oil in a quarter of an hour and soon become very pronounced. In an hour the whole fish will have become pale and it will remain so for about a day even in a black-walled, illuminated vessel. After this the fish will darken wholly except for the pale spot which will remain obvious for some 3 to 4 weeks when it will finally disappear. Injections of adrenalin in oil 1:1,000 to 1:100,000 are all followed by the formation in the fish of pale spots above the

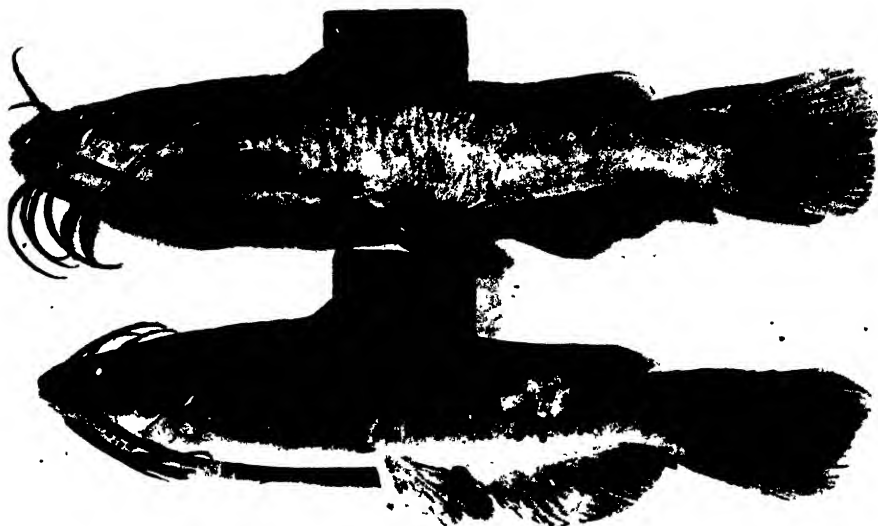


FIG. 1. Photographic reproduction of two prepared catfishes, *Ameiurus nebulosus*, the upper one pale, the lower one dark. On the upper flank of the pale fish at the front edge of its adipose fin is a dark mottling due to the dispersing action on the melanophore pigment of one part of acetylcholine in 20 parts of olive oil injected anteriorly from a point a little behind the darkened area. In a corresponding position on the dark fish is a strikingly pale area irregular in outline and due to the concentrating action of adrenalin one part in 1000 of oil injected anteriorly from the posterior edge of what subsequently became the pale area itself.

oil but without general blanching (Fig. 1). Such spots may persist in the cases of the stronger mixtures for some 3 weeks and in the weaker ones for about 2 weeks after which they vanish. Injections of 0.1 cc. of adrenalin 1:100,000 of oil were never followed by color changes of any kind in the fishes. So far as change of color was concerned this mixture was fully inactive as is pure olive oil.

When 0.1 cc. of a mixture of adrenalin 1:100 of olive oil is injected subcutaneously into the flank of a dark catfish a pale spot, as already stated, quickly appears over this oil. The pigment in the melanophores of this spot, as can

be seen under the microscope, is concentrated to the maximum degree and appears as a minute black dot in each color cell (Parker, 1941). Soon the whole fish becomes pale. The formation of the pale spot and the blanching of the fish is believed to be due to the escape from the oil of some of its contained adrenalin into the surrounding lymph and the transfer of this neurohumor thus through lymph and blood to the melanophores in general. This operation is most effective immediately after an injection of oil and adrenalin has been made. When the general blanching has begun to subside, as happens in a day or so, it gives way to normal darkening and the pale spot on the flank of the fish is the only blanched area that remains. The life of this spot is apparently dependent upon the amount of adrenalin still in the oil. Enough of this neurohumor appears to be present in the kind of injection here described to cause the spot to persist in some instances for as long as 3 weeks. This general explanation of the color changes thus artificially induced in catfishes is consonant with the observations that with weaker concentrations of adrenalin in oil no general blanching appears but only pale spots are to be seen and further that the weaker the concentration of adrenalin the shorter the life of such a spot. The oil appears to serve as a reservoir for the adrenalin which may thus gradually escape into the surrounding tissues until this neurohumor is exhausted. An injection of a strong, aqueous solution of adrenalin into a catfish will blanch the fish quickly and fully, but this response will last only a part of a day whereas a spot formed by adrenalin in oil may persist several weeks. This method of administering adrenalin has already found its place in medicine, for physicians who wish to extend the action of this drug over long periods as in cases of asthma do so by injecting it hypodermically in oil.

### 3. *Acetylcholine*

The acetylcholine used in these tests was the chloride, a white, finely crystalline salt manufactured by the Swiss firm of Hoffmann-La Roche and Company. It was kept hermetically sealed in glass tubes, 0.1 gm. in a tube, and mixed with oil or water from day to day as needed. Although injections of extremely dilute mixtures of acetylcholine in Ringer's solutions will darken catfishes (0.2 cc. of a mixture of one part acetylcholine in a thousand-million parts of solvent), this neurohumor in oil is similarly effective only in surprisingly concentrated states. Injections of 0.1 cc. of a mixture of acetylcholine and olive oil 1:1,000 or 1:10,000 have little or no effect upon the melanophores of *Ameiurus*. Indubitable local darkening of this fish occurs only when the oil contains about 1 per cent of acetylcholine or better 5 per cent. In such a mixture much of the acetylcholine is in suspension. As it reaches the surface of the oil after the mixture of oil and neurohumor has been injected it presumably dissolves freely in the surrounding watery fluid of the fish's tissues.

This fluid is believed to be rich in cholinesterase which rapidly destroys the acetylcholine and thus prevents it from reaching the melanophores. Hence only very concentrated mixtures of acetylcholine in oil give out a sufficiency of this agent to survive long enough in the cholinesterase environment to reach and affect the color cells. The probable correctness of this view is seen from the fact that if a catfish that has received by injection oil containing 5 per cent acetylcholine is injected further with an appropriate amount of eserine by which the neurohumor may be protected from the destructive cholinesterase, the fish will quickly darken as a whole and soon die. Thus the acetylcholine stored and protected in the oil may spread without destruction to the melanophores, darken the fish as a whole, and eventually poison it. It is interesting to note that when 0.1 cc. of even 1 per cent acetylcholine in oil is injected into a catfish a total amount of this agent is received by the fish sufficient to kill it outright. The oil, however, allows this lethal dose of acetylcholine to escape into the watery tissues of the fish only so slowly that the neurohumor is destroyed by the local cholinesterase before it can exert its deadly effect. Biologically the fish may be said thus to protect itself against a poison, acetylcholine, that is a product of its own tissues.

When the dark spots produced on a catfish by the injection of 5 per cent acetylcholine in oil are examined under the microscope, their melanophore pigment is seen to be only about half dispersed. This is the characteristic response of catfish melanophores to acetylcholine. It is as well marked in the artificial type of stimulation as under normal conditions (Parker, 1941). Such dark spots, particularly if they have been induced by 5 per cent acetylcholine in oil, will remain visible for 3 to 4 days, but they never show the length of life of the pale spots due to adrenalin in oil, some 3 to 4 weeks.

#### *4. Storage of Neurohumors in the Skin of the Catfish*

That acetylcholin can be actually stored for some days in the skin of a catfish may be inferred from the following experiment.

As a preliminary step a drop of olive oil containing 5 per cent of acetylcholine was injected into the flank of a catfish of intermediate tint. A quarter of an hour later well marked, small, dark areas were visible in the fish's skin over the oil (Fig. 1). Half an hour after the first injection 0.2 cc. of adrenalin 1:100,000 of Ringer's solution was injected into the fish. This was soon followed by a general blanching of the fish including the small, dark areas. The next day, 20 hours after the initial injection, the fish was palish and over the region of the oil faint but well marked, dark spots were to be seen again. A new injection of 0.2 cc. of adrenalin 1:100,000 of solvent was then made, whereupon in a short time the dark spots again disappeared and the whole fish was pale. Some 12 hours later the spots, now very faint, reappeared, but in the course of the day they vanished of themselves.

Three fishes in all were tested in this way. One of them was in all essentials a duplicate of that just described. In the other the dark areas after having disappeared under adrenaline returned once but not a second time. Thus it is possible to obliterate the dark areas produced by acetylcholine in oil through the use of adrenalin as a hydrohumor and still have the dark areas return once or twice. Such a return points very clearly to a storage of acetylcholine in the oil over the period during which the melanophore pigment is concentrated by adrenaline.

The converse experiment can be performed with adrenalin and intermedin. If a catfish of intermediate tint with a pale spot produced on it by a small amount of adrenalin 1:1,000 in oil is injected with intermedin, the whole fish will rapidly darken and the pale spot will disappear. After about a day and without further treatment the pale spot will return, showing that the adrenalin is still active and that it was only temporarily subdued. Apparently for a few hours after the injection of the intermedin the concentration of this neurohumor about the melanophores of the pale spot was so great as to overcome the action of the adrenalin from the oil and thus to induce a temporary dispersion of the melanophore pigment. Finally, with the gradual disappearance of the intermedin the adrenalin reasserts itself and the pale spot reappears. Such an interplay with antagonism between neurohumors has already been pointed out and discussed in considering the color changes of the smooth dogfish *Mustelus* (Parker, 1937).

In the catfish this type of alternating change in color where blanching from adrenalin in oil is temporarily obliterated by intermedin can be repeated a number of times. In one fish of intermediate tint in which a pale spot had been produced by the injection of 0.1 cc. of adrenalin 1:10,000 of oil this spot in the course of 3 days was made to disappear by injections of aqueous intermedin and then reappeared of itself no fewer than seven times. Such an instance demonstrates the great effectiveness of the protection given by the oil to the adrenalin and the considerable length of time over which this protection may extend.

Artificial protection of adrenalin by oil such as has been described supports the view that persistence of the pale phase shown in the first few darkenings of catfish previously kept blanched for a considerable time in a white environment is due to a storage of the blanching agent, doubtless adrenalin, before its final disappearance. The gradual shortening of the pale periods may be taken as a measure of the exhaustion of this reserve of adrenalin. A similar type of response appears to be true of acetylcholine.

It is well known that the nerve terminals for melanophores are not in the least like those of muscle fibers. In place of the one or at most a few nerve fiber branches to a muscle fiber scores of nerve terminals surround each melanophore. Some of these must be concentrating, others dispersing. A number

of these terminals are applied to the melanophore but others end some distance from it. Such an arrangement agrees well with what has been said about melanophore activation. In those instances where a nerve terminal is in direct contact with a color cell the activating neurohumor may pass directly from the terminal to the cell, but in the others where the branches of the nerve fibers end at some distance from the cell the discharge of the neurohumor may well be into the tissue lipoids from which the neurohumor may then pass by way of the pericellular lymph to the melanophore. This course is the one in which as a result of the interpolation of the lipid stage neurohumoral storage may occur. Thus in this instance the conditions are present for inducing retardation in the first few responses in a melanophore which for a relatively long time has been held in a color phase opposite to that toward which it is moving.

#### SUMMARY

1. Adrenalin when mixed with olive oil 1:100 and injected subcutaneously into a catfish will produce a marked pale spot and a general blanching of the whole fish. The general blanching will disappear in about a day. The pale spot may last 3 to 4 weeks.

2. Injections of adrenalin in oil 1:1000 to 1:10,000,000 cause the formation of only pale spots in catfishes. Adrenalin in oil 1:100,000,000 has no effect on the fish's color.

3. Acetylcholine, though ordinarily more powerful than adrenalin, when mixed with oil is effective only in high concentrations 1:20 to 1:100. It then produces clusters of moderately dark spots.

4. The pale spot on a catfish due to adrenalin in oil may be temporarily obliterated by intermedin. It will return of itself, and this process may be repeated a number of times. The same is true of the dark spots produced by acetylcholine in oil when subjected to adrenalin. These observations indicate the subcutaneous storage of both adrenalin and acetylcholine in oil.

5. In the natural color changes in catfishes the nervous neurohumors, probably also adrenalin and acetylcholine, appear both to be stored in the fish's lipoids and to serve thus as a means of lengthening the times of initial color responses till these stores of neurohumors are exhausted.

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## INACTIVATION OF PEPSIN BY IODINE

### II. ISOLATION OF CRYSTALLINE *l*-MONO-IODOTYROSINE FROM PARTIALLY IODINATED PEPSIN

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In a previous paper (Herriott, 1937) evidence was presented to show that iodine reacts with the tyrosine of pepsin and causes inactivation of the enzyme. After complete iodination and inactivation over 80 per cent of the iodine was recovered as di-iodotyrosine. Philpot and Small (1939) found that a small amount of iodine caused some inactivation of pepsin without any measurable drop in Folin's colorimetric tyrosine test and suggested, therefore, that some group other than tyrosine was involved. In the present work small amounts of iodine were introduced into the pepsin molecule and 65 per cent of this iodine was subsequently identified as *l*-mono-iodotyrosine. No evidence was obtained for any other iodine compound. The fact that mono-iodotyrosine has about 80 per cent of the molar color value of tyrosine instead of the 50 per cent of di-iodotyrosine explains the results of Philpot and Small.

The iodinated pepsin preparations were found to be fairly homogeneous as judged by a number of fractionation experiments. Crystallization of the iodinated pepsin and a subsequent solubility experiment showed the material to be very different from pepsin and to be relatively pure. The crystals were microscopically indistinguishable from those of pepsin. Electrophoresis failed to separate a mixture of pepsin and iodinated pepsin.

#### *Iodination of Pepsin*

Purified pepsin solutions (for preparation see Experimental Methods) were mixed with dilute alcoholic solutions of free iodine or dilute iodine in KI at pH 5.0-6.0 and room temperature until the iodine disappeared from solution. The iodine concentration had been adjusted so that there was a loss of only 10-20 per cent of the activity and no measurable change in the blue color value with Folin's phenol reagent. Half the free iodine that had disappeared from solution was found to be organically bound to the protein which is just what one would expect of a substitution reaction of iodine. Oxidation by iodine under these conditions is therefore improbable. This iodinated pepsin con-



tained about 0.7 per cent iodine or only 2 iodine atoms per molecule of pepsin. This amount is only one-twentieth of the amount of iodine previously found necessary to completely inactivate and completely iodinate pepsin. Iodide

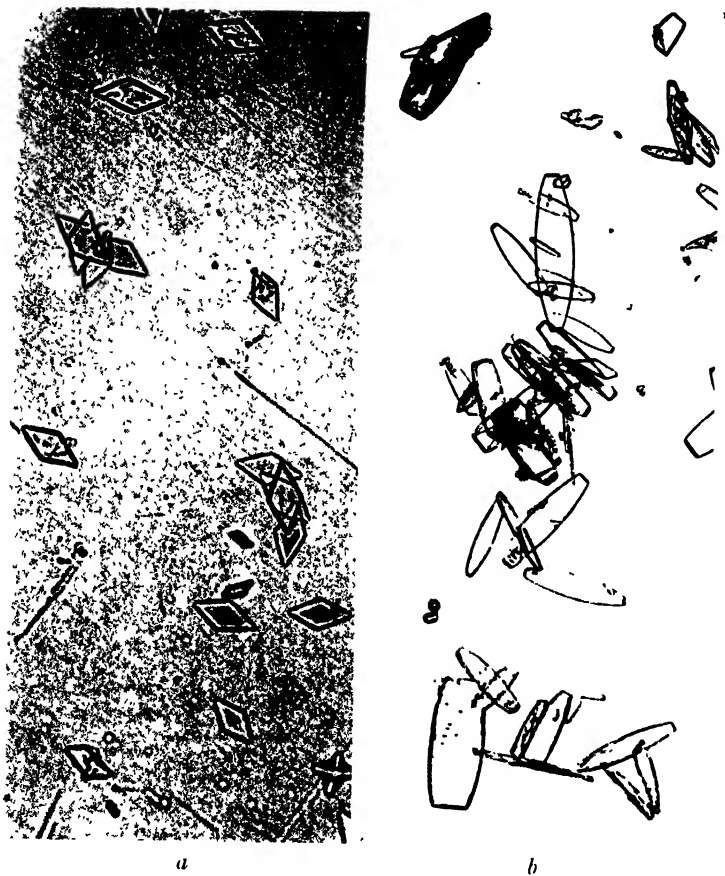


FIG. 1 *a*. Crystalline *l*-mono-iodotyrosine from iodinated pepsin.

*b*. Crystalline di-iodotyrosine from iodinated pepsin.

ion was removed by dialysis or by washing the denatured protein precipitate on a funnel with  $N/100$  sulfuric acid.

#### *Crystallization of Iodinated Pepsin*

Crystallization of iodinated pepsin was carried out in much the same way as for the original pepsin, *i.e.* precipitation by titrating to pH 2.5; stirring the filter cake in one-half its volume of water at 35° C. plus just enough  $N/2$  sodium hydroxide to dissolve the solid, then stirring and cooling slowly.

The resulting crystals were indistinguishable under the microscope from ordinary pepsin crystals. Only one preparation was crystallized. No other attempts were made since there was no indication of any change in properties by crystallization.

### *Tests of Purity*

It was thought important to determine whether the iodinated pepsin preparations were reaction mixtures of several proteins of varying iodine content or if the material was fairly homogeneous. This would have some bearing on the question as to the amount of iodine necessary to inactivate a pepsin molecule.

TABLE I  
*Fractionation Experiments*

Sample No	Materials and Procedure	$\frac{[P.U.]_{mg}^{Hb}}{P.N.}$	Iodine
			per cent
1	Original pepsin	0.32	
2	No. 1 after iodination*	0.23	1.4
3	A fraction of No. 2 insoluble in 0.5 sat. $MgSO_4$ 0.05 M pH 4.6 acetate buffer	0.22	
4	First crystals of No. 2	0.23	1.4
5	Mother liquor from first crystals	0.22	1.3
6	Fraction of No. 4 soluble in 0.25 M $Na_2SO_4$ pH 4.0	0.21	1.45
7	Fraction of No. 4 that did not dissolve in aliquot of 0.25 M $Na_2SO_4$ -pH 4.0 used in No. 6	0.21	1.0

\* More iodine was added to this particular preparation than to the others which accounts for its higher iodine content and lower specific activity  $[P.U.]_{mg}^{Hb}/P.N.$

Iodinated pepsin preparations were therefore subjected to various fractionating tests and the various fractions analyzed.

It is apparent from the results in Table I and Fig. 3 that the preparations are relatively homogeneous and do not consist of an inactive iodinated protein mixed with some of the original pepsin. It is likely then that the iodine distributes itself fairly evenly among all the protein molecules.

**Electrophoresis.**—Electrophoresis of a solution of the crystalline iodinated pepsin in the Tiselius cell (kindly analyzed by Dr. Alexandre Rothen) at pH 4.4 revealed that there was only one moving boundary. However, when this same material was mixed with equal amounts of pure pepsin the mixture also showed only a single sharp boundary both before reversing the current and afterwards (Fig. 2). This was done at only one pH and it is possible that at some other pH they could be separated. The mobilities were as follows: for the ascending

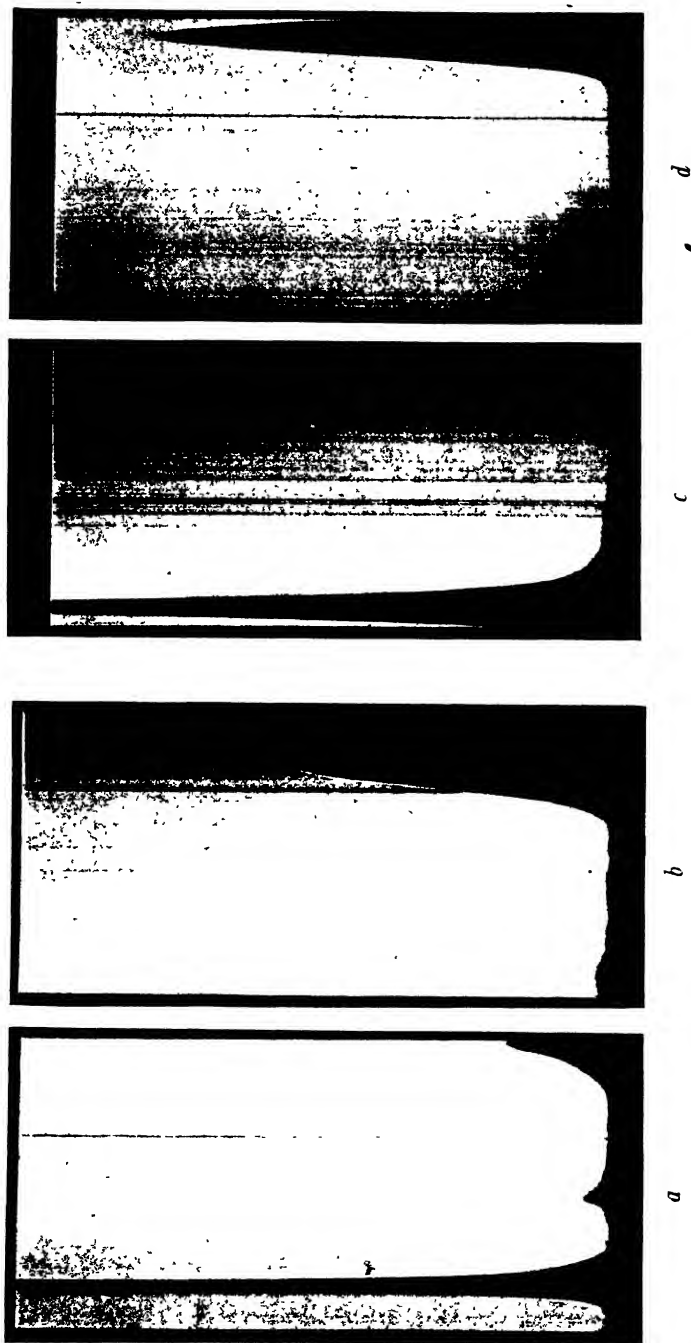


FIG. 2. Electrophoresis patterns of an artificial mixture of pepsin and iodinated pepsin at pH 4.4; specific conductivity 0.00069 reciprocal ohms. *a* and *b* are the ascending and descending boundaries after 2460 seconds; *c* and *d* are the ascending and descending boundaries after reversing the direction of the current and running for 2640 seconds.

boundary of the iodinated pepsin  $8.1 \times 10^{-5}$  and the descending boundary  $7.8 \times 10^{-5}$ . In the mixture of iodinated pepsin and pepsin the ascending boundary was  $7.8 \times 10^{-5}$  while the descending boundary was  $7.5 \times 10^{-5}$ , all values being expressed in  $\text{cm}^2/\text{volt}/\text{sec}$ .

**Solubility Curve.**—A solubility curve of the crystalline pepsin is shown in Fig. 3. In the solvent used, 0.25 M sodium sulfate pH 4.0, crystalline pepsin is at least 20 times as soluble as the crystals of iodinated pepsin. The two proteins can therefore easily be distinguished. The curve in Fig. 3 is not that of an ideal substance but it shows no great degree of inhomogeneity. The points marked  $\circ$  and  $\square$  are values determined and calculated when some pepsin was added to the solvent before adding the crystalline iodinated pepsin. The value  $\square$  was expected only if the solubilities of the two proteins were independent of each other; *i.e.*, they form a mixture. Since the value fell considerably below

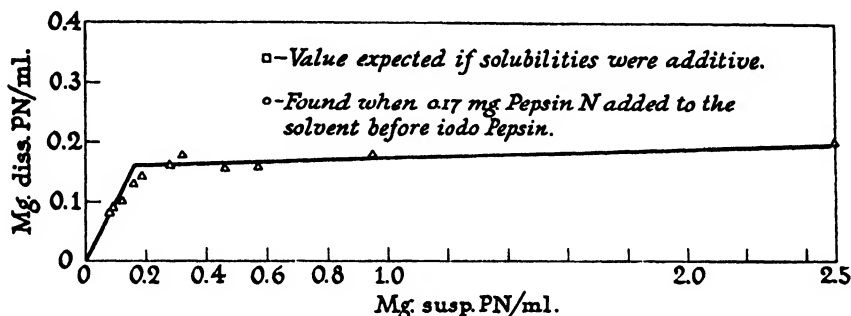


FIG. 3. Solubility of crystals of iodinated pepsin in 0.25 M  $\text{Na}_2\text{SO}_4$  pH 4.0 at 23°C.

the expected, it may be concluded that the iodinated pepsin forms solid solutions with ordinary pepsin.

The solubility of iodinated pepsin will probably decrease as more iodine is introduced into the molecule.

#### *Isolation of l-Mono-Iodotyrosine from Iodinated Pepsin*

The experimental procedure for the isolation of pepsin and subsequent isolation of *l*-mono-iodotyrosine is shown in Table II. Fig. 1 *a* shows the appearance of the crystalline *l*-mono-iodotyrosine. In one preparation a small amount of di-iodotyrosine was also crystallized out (Fig. 1 *b*).

As may be seen in Table II, solution No. 13, contains 65 per cent of the original iodine. This was successively extracted with butyl alcohol until over 80 per cent of the total iodine had been extracted. The fraction extracted by each aliquot of butyl alcohol was reasonably constant over the whole range as may be seen in Fig. 4. Under similar conditions the fraction of di-iodotyrosine extracted is 50 per cent instead of the 20 per cent, as shown in Fig. 4. It could

TABLE II  
*Isolation of L-Mono-Tyrosine from Iodinated Pepsin*

Materials and procedures	No.	Vol.	Total N	Total I	Phenol <sup>a</sup> I	I <sup>b</sup> N	Phenol <sup>c</sup> N
		ml.	mg.	mg.			
Dialyzed purified pepsin solution, pH 5.3 .....	1	1075	5700				
No. 1 + (20 ml. 4 M NaAc to pH 5.7 + 5.5 ml. N/1 alcoholic iodine) slowly at 15°C. Allowed to stand until colorless. The specific activity had dropped 11 per cent. ....	2	1100					
No. 2 + 5 ml. 5 N NaOH to pH 7.0, warmed to 60°C. for 5 min. then + 50 ml. 5 N H <sub>2</sub> SO <sub>4</sub> and filtered and washed twice with 100 ml. of M/100 H <sub>2</sub> SO <sub>4</sub> . Residue dissolved in NaOH + H <sub>2</sub> O. .	3	500	5650	300			
No. 3 + 200 gm. Ba(OH) <sub>2</sub> crystals and refluxed 20 hrs., then cooled and left 50 hrs. at 5°C. Filtered and residue washed. Filtrate .....	4	650		310			
No. 4 heated to 90°C. then + 115 ml. 5 N H <sub>2</sub> SO <sub>4</sub> with stirring to pH 3.5 and aerated for 1 hr. to remove H <sub>2</sub> S. 200 ml. M/1 PbAc <sub>2</sub> added and solution filtered and residue washed three times with 80 ml. each of N/10 acetic acid. Filtrate and washings. ....	5	1300		307			
No. 5 + 85 ml. 5 N NaOH pH 8.5; let stand, filter, and wash on funnel. Filtrate and washings .....	6F	1400		56			
Precipitate .....	6P						
No. 6P stirred with dilute acetic acid to pH 4.0, then + HCl to pH 2.0, filter. Filtrate diluted to 1 liter; precipitate appeared which was filtered off and discarded. Filtrate (pH 4.3). Titrated to pH 8.0 and filtered. Filtrate. ....	7F	1000		16			
Precipitate. ....	7P						
No. 7P + dilute acetic acid to pH 4.0. Filtered. Filtrate. ....	8F						
Precipitate. ....	8P						
No. 8P + HCl to dissolve, then + NaAc to pH 4.0; filter. Filtrate. ....	9F	50	4.5	24	1.15	5.3	6.2
No. 8F + alkali to pH 8.0, filter and wash residue. Filtrate + washings. ....	10F	500		10			
Precipitate. ....	10P						
No. 10P + H <sub>2</sub> SO <sub>4</sub> to pH 3.0, filtered and washed with H <sub>2</sub> O. Filtrate + washings. ....	11F	85	23.5	150	1.4	6.5	9.1
Precipitate. ....	11P						

TABLE II—Continued

Materials and procedures	No.	Vol.	Total N	Total I	Phenol <sup>a</sup> I	If N	Phenol <sup>b</sup> N
		ml.	mg.	mg.			
No. 11P ground in mortar with N/20 H <sub>2</sub> SO <sub>4</sub> , filtered. Filtrate.....	12	42	7.1	29	1.6	4.0	6.2
No. 9F + No. 11F + No. 12 concentrated <i>in vacuo</i> .....	13	84	33.5	193	1.5	5.75	8.8
No. 13 diluted to 100 ml. with dilute H <sub>2</sub> SO <sub>4</sub> , pH 2.5 then + 50 ml. butyl alcohol saturated with water and shaken 3 min. in separatory funnel.							
Separate. Butyl alcohol layer.....	14A	38.5		49	1.6	6.6	10.5
Water layer.....	14W	111.0		145			
No. 14W + 38 ml. butyl alcohol saturated with H <sub>2</sub> O, shaken, and separated.	15A	38		39			
	15W	111		106			
No. 15W + 38 ml. butyl alcohol saturated with H <sub>2</sub> O, shaken, and separated.	16A	38		23			
	16W	110		83			
No. 16W + 38 ml. butyl alcohol saturated with H <sub>2</sub> O, shaken, and separated.	17A	38		17.5	1.2	7.4	8.9
	17W	108		66			
No. 17W + 38 ml. butyl alcohol saturated with H <sub>2</sub> O, shaken, and separated.	18A	38		13.7			
	18W	104		52			
No. 18W + 38 ml. butyl alcohol saturated with H <sub>2</sub> O, shaken, and separated.	19A	39		10			
	19W	103		43			
No. 19W + 38 ml. butyl alcohol saturated with H <sub>2</sub> O, shaken, and separated.	20A	39					
	20W	102					
No. 20W + 38 ml. butyl alcohol saturated with H <sub>2</sub> O, shaken, and separated.	21A	39		6.8	1.5	6.8	10.0
	21W	101		28.6	2.7	2.1	5.7
All of the butyl alcohol solutions from 14A–21A collected and evaporated <i>in vacuo</i> to 50 ml. This was then extracted with 25 ml. saturated Ba(OH) <sub>2</sub> solution + 25 ml. water. Alkali water layer.....	22	48		20			
No. 22 + H <sub>2</sub> SO <sub>4</sub> to pH 3.0, filter and wash residue; evaporate to 10 ml. then + 5 N NaOH to pH 3.5, let stand at 5°C.; a small brown precipitate came out, was centrifuged off, and discarded. Titrate supernatant to pH 5.7 and let stand. Rosettes of diamond-shaped platelets appeared. Place in shallow dish and evaporate to 2.3 ml., cool, filter.							
Filtrate + washings.....	23F	1.5		22.5			
Crystalline precipitate.....	23P						

TABLE II—*Concluded*

Materials and procedures	No.	Vol.	Total N	Total I	Phenol* I	I† N	Phenol‡ N
		ml.	mg.	mg.			
No. 23P dissolved.....	24	12.5	9	88	1.0	10.0	9.6
No. 21W + various residues + washings.....	25	22		57			
No. 25 extracted twice with 50 ml. butyl alcohol.....	26A 26W	115 15		55 2			
No. 26A extracted with Ba(OH) <sub>2</sub> ; acidify water layer with H <sub>2</sub> SO <sub>4</sub> , centrifuge; supernatant + 23F evaporated down. Diamond platelets again formed; Filter and wash residue once.							
Filtrate.....	27F	5.5		25	2.7	2.8	7.6
Crystals dissolved.....	28P	9.3		37	1.8	6	10.8
No. 24 + No. 28P.....	29			125			
No. 29 recrystallized.							
Crystals dissolved.....	30P	9	10	100	1.13	9	10
Mother liquor.....	30F	6.5	8	30	1.9	4.1	7.7

\* This ratio is number of milligrams of tyrosine that give the equivalent color with Folin's phenol reagent divided by the number of milligrams of iodine.

† This ratio is the number of milligrams of iodine of a sample divided by the number of milligrams of nitrogen.

‡ This ratio is the same equivalent color as in the first footnote (\*) but divided by the number of milligrams of nitrogen.

be concluded from this distribution experiment, therefore, that the iodine-containing component was probably not di-iodotyrosine and that the component was fairly homogeneous with respect to iodine; *i.e.*, there was only one iodine component. It should be pointed out that the analyses of the fraction in the first butyl alcohol extract, 14A of Table II, are practically the same as the analyses of the last extract, 21A. This is additional evidence regarding the homogeneity of this material. In Fig. 4 the ordinate scale spacing is logarithmic.

Although only 42 per cent of the original iodine was obtained as crystalline mono-iodotyrosine, the distribution or extraction experiment makes it highly probable that over 65 per cent of the original iodine was present as iodinated tyrosine.

The details of the distribution experiment are given in Table II but a few comments are appropriate. Butyl alcohol and water have an appreciable solubility in each other. Therefore for constancy of volume it is necessary to saturate each with the ether. In Table II, No. 14 of the water solution was not initially saturated with butyl alcohol but after the first extraction with 50 ml. of butyl alcohol the butyl

alcohol volume decreased to 38 ml. and the difference was the volume of butyl alcohol necessary to saturate the water layer. To simplify the problem, therefore, in the second and succeeding extractions 38 ml. of butyl alcohol saturated with water was used. By doing this the volumes remained constant.

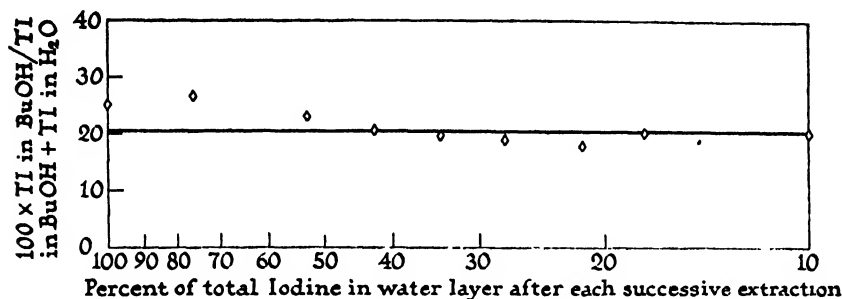


FIG. 4. Distribution of iodine components between butyl alcohol and water at pH 3.0 and 25°C. on successive extractions.

#### *Identification and Properties of l-Mono-Iodotyrosine*

Mono-iodotyrosine was isolated only recently by Ludwig and Mutzenbecher (1939) from iodinated casein. They measured very few properties but state that, as compared to di-iodotyrosine the mono- derivative had the same crystalline appearance, was more soluble in water, had the same melting point, and, of course, differed primarily in its iodine content.

Table III shows the results we have obtained compared to those of di-iodotyrosine. On one occasion the mono- derivative crystallized out with rounded edges appearing somewhat like the whetstones of di-iodotyrosine but most of the time they appeared as sharply formed diamond platelets as shown in Fig. 1 *a* or rosettes of these platelets.

The iodine and nitrogen values obtained are slightly different from those reported by Ludwig and Mutzenbecher but they found one molecule of water of crystallization in their product. Since our product was dried at 70°C. *in vacuo* this water was presumably driven off, thus accounting for the discrepancy. Our values agree quite closely to the theoretical percentages of an anhydrous material.

In view of the fact that Ludwig and Mutzenbecher had also obtained thyroxine from iodinated casein it was thought that possibly our crystalline iodine derivative might be related to thyroxine perhaps as an ether of one molecule of ordinary tyrosine and one of di-iodotyrosine. Such a compound would yield elementary analyses close to those of mono-iodotyrosine. However, a molecular weight determination by the Barger vapor pressure method (1904) using formic acid as the solvent and di-iodotyrosine as the standard showed the



compound to have a molecular weight in the neighborhood of 300 which rules out the ether possibility.

The moniodo derivative gives a strong Millon's reaction so that one should be careful of concluding that tyrosine has not been iodinated merely from a positive Millon reaction even though carried out quantitatively.

TABLE III  
*Properties of Crystalline l-Mono-Iodotyrosine Compared to Di-Iodotyrosine*

Property	Monoiodo-	Diiodo-
Crystalline form.....	diamond platelets	whetstones or needles
$[\alpha]_D$ in 4 per cent HCl at 22°C. ....	-8.8	-2.9*
Solubility in water in mg./ml. ....	4	0.8
Melting or decomposition point .....	201°C. corrected	195°C. corrected
Phenol color value/iodine, ratio† .....	1.1	0.3
Phenol color value/nitrogen, ratio† .....	10.0	6.2
Iodine/nitrogen, ratio†.. ....	9.0	18.0
Reaction to Millon's reagent.. ....	+	-
Nitrous acid color test for ortho-iodo-benzene derivatives†.....	+	+
Per cent iodine.....	42.0 found§ 41.5 calculated	
Per cent nitrogen.....	4.5 found§ 4.55 calculated	

\* Value from Abderhalden (1923)

† These ratios are explained in the footnotes of Table II

‡ Kendall and Osterberg (1919)

§ The sample used for these analyses was dried 15 hours at 70°C. *in vacuo*.

#### EXPERIMENTAL METHODS

The extraction procedure consists of agitation by hand in a separatory funnel for 3 minutes at room temperature after which the solutions are allowed to separate and each layer drawn off and analyzed for total iodine. The pH of the water solution was adjusted at the start to between pH 2.3 and pH 3.3 with sulfuric acid.

*Preparation of Pepsin.*—The pepsin used through out this work was prepared according to Table VI of a previous publication (Herriott, Desreux, and Northrop, 1940). The protein in the filtrate No. 4 of that table was precipitated by addition of 250 gm. of solid  $MgSO_4 \cdot 7H_2O$  for every liter of filtrate. The precipitate was filtered off and dialyzed in cellophane bags against tap water overnight.

*Determination of Nitrogen.*—This estimation was described by Northrop and Kunitz (1932). Instead of distilling into hydrochloric acid as previously done, a 4 per cent boric acid solution with brom cresol green indicator is used. Only one titration is required and this is with N/70 hydrochloric acid. This change was brought to the writer's attention by Dr. Bacon F. Chow.

*Determination of Iodine.*—A modification of the method of Kendall, as previously described by Herriott (1937) was used.

**Determination of Phenol Color.**—1 ml. of a solution of material which would yield about the same intensity of blue color as 0.15 mg. of tyrosine was put into a 50 ml. Erlenmeyer flask; 8 ml. of 0.5 N sodium hydroxide was added, followed by 3 ml. of a 1/3 dilution of Folin's phenol reagent prepared according to Folin and Ciocalteu (1927). The phenol reagent was added dropwise with stirring and the color read after 10 minutes against a tyrosine standard treated in the same way or a calibrated blue glass. When the unknown contains a high concentration of buffering material more alkali is required.

**Peptic Activity Measurements.**—The hemoglobin activity method was that of Anson (1938). The rennet activity method was that of Herriott (1938).

**Solubility Methods.**—This has been discussed in detail by Herriott, Desreux, and Northrop (1940).

#### SUMMARY

1. Pepsin solutions were iodinated at pH 5.0–6.0 until 10–20 per cent of the activity was lost and 1/20 (0.7 per cent) of the saturating amount of iodine had been introduced into the protein molecule. After alkaline hydrolysis 65 per cent of the original iodine was accounted for as mono-iodotyrosine although only 42 per cent was isolated as a crystalline product. No evidence was obtained to support the possibility that any group other than tyrosine in pepsin was iodinated.

2. Some of the properties of the crystalline *l*-mono-iodotyrosine were determined and compared to those of di-iodotyrosine.

3. One iodinated pepsin preparation was crystallized. The crystal form was the same as that of the original pepsin. A solubility curve of the crystals demonstrated that it was very different from pepsin and had nearly constant solubility.

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# THE OCCURRENCE AND DISTRIBUTION OF ATROPINESTERASE, AND THE SPECIFICITY OF TROPINESTERASES\*

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The term "tropine esterase" was first employed in a previous communication (1) for convenience in designating the enzyme effecting the hydrolysis of atropine and related structures. The contracted form, tropinesterase, will be used henceforth since it has become customary to employ the analogous contraction, cholinesterase. In the present investigation, which includes a study of a variety of tropine esters, the possibility has arisen that the tropinesterase acting on one of the compounds may not be the same as that effective on another. In a case of this nature, when there is reason to emphasize the particular substrate being affected, more explanatory terms such as atropinesterase or cocainesterase will be employed in a manner similar to the use of the long established terms, methylbutyrase or tributyrinase. It now appears that the term, atropinesterase, rather than the more general, tropinesterase, would have been more appropriate in the previous paper (1) which was concerned exclusively with atropine hydrolysis.

In the previous study the effects of enzyme and substrate concentrations, pH, and temperature were investigated with regard to the enzymatic hydrolysis of atropine by rabbit serum. This work has been extended in the present paper to include the occurrence and distribution of atropinesterase in the rabbit, the scission of atropine and homatropine by the serum and liver of the guinea pig and frog, the effect of fresh egg white and yolk upon atropine, and the specificity of tropinesterases.

## EXPERIMENTAL

The measurements of enzyme activity were carried out by the manometric method employing the Warburg apparatus in a manner essentially the same as that already described (1). Corrections for non-enzymatic hydrolysis were made in the usual fashion. As employed in the present study, the tropinesterase unit may be defined as the quantity of enzyme required to liberate 1 c.mm. CO<sub>2</sub> in 300 minutes at 30° in a total volume of 4 ml. in the bicarbonate-Ringer medium containing a concentration of substrate sufficient to achieve the maximum rate of hydrolysis. For studies of atropine splitting a final concentration of 0.25 per cent atropine sulfate was used,

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except in the specificity studies. The tropinesterase value, employed to express the enzyme concentration, is defined as the number of units per 100 mg. of material.

Readings of the manometers were taken every 30 minutes and the hydrolytic rate was obtained from the slope of the line representing the  $\text{CO}_2$ -liberation: time relationship. In cases where the gas evolution proceeded at an initially accelerated rate

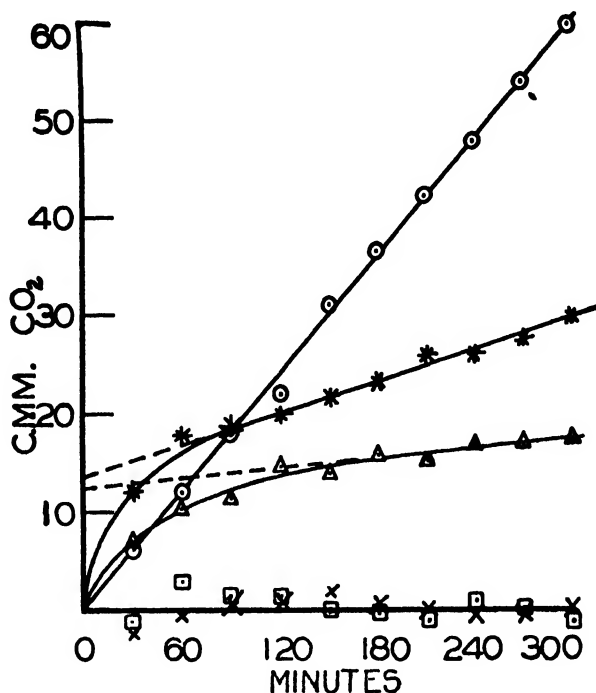


FIG. 1. Hydrolysis of atropine and homatropine by guinea pig liver.

(X) Control experiment with liver alone.

(Δ) Control experiment with atropine alone.

(\*) Control experiment with homatropine alone.

(◻) Action of liver on atropine (corrected for non-enzymatic hydrolysis).

(○) Action of liver on homatropine (corrected for non-enzymatic hydrolysis).

Broken lines are extrapolations of linear portions of curves. Half the usual volume of all constituents used (2 ml. total volume).

before attaining linear proportions, as in the instance of the non-enzymatic hydrolysis of atropine and homatropine (Fig. 1), only the linear portion of the curve was employed to determine hydrolytic rate. However, each point in the non-enzymatic curves, whether or not it fell in the linear portion, was subtracted from the corresponding value for total hydrolysis in order to obtain the enzymatic gas liberation.

For the specificity studies, 1 per cent substrate concentrations were found to be sufficient to insure maximum hydrolytic velocities in all cases despite the differences

in the molecular weights of the esters tested. All substrates were used in the form of their salts, and were products of Merck & Co. with the exception of novatropine which was obtained from Campbell Products, Inc.

The cholinesterase measurements were carried out in a manner similar to that used for tropinesterase; a final substrate concentration of 0.375 per cent acetylcholine chloride was employed unless otherwise stated, and readings were taken every 10 minutes during a total reaction period of 60 minutes. To obtain values comparable with those for tropinesterases the 60 minute values were extrapolated to 300 minutes.

Tissue extracts for enzyme study were prepared by grinding the material with cleaned and ignited sand, adding bicarbonate-Ringer solution until a final volume of 10 ml. for each gram of tissue was obtained, and centrifuging the suspension. The supernatant fluid was decanted and used.

For the investigation of tropinesterase distribution in the cells and serum of defibrinated blood, 10 ml. portions of the latter were centrifuged yielding 6 ml. of serum and 4 ml. of cells in each case. In order to obtain comparable data, the 6 ml. of serum were diluted to 10 ml. with distilled water, and the cells, after being washed twice with physiological saline solution, which was discarded each time after centrifuging, were also made up to 10 ml. with distilled water. The serum and cell solutions were further diluted 10 times with water before use; the whole defibrinated blood was also diluted 10 times with water before use. Water was employed for dilution to hemolyze the cells.

## RESULTS

The examination of the sera of sixty-eight rabbits chosen at random revealed that only nineteen were enzymatically active in the hydrolysis of atropine. The atropinesterase values obtained varied from 51 to 270, average 107. Both active and inactive sera were obtained from animals during all seasons of the year, from the albino variety as well as those of various colors, from animals of both sexes, and all ages and weights. There was no correlation between the atropinesterase and cholinesterase values. All of the rabbits possessed cholinesterase activity, and the values for the sera of fifteen animals, which also contained atropinesterase, varied from 140 to 300, average 205; those from thirty-three animals devoid of atropinesterase fell between 110 and 290, average 210.

Mixing a serum containing atropinesterase with an equal volume of serum devoid of activity was found to have no influence on the enzyme in the former. Thus, 98 enzyme units were found in 0.1 ml. of active serum, and 97 units in 0.2 ml. of the mixture.

The distribution of atropinesterase in defibrinated blood from rabbits possessing activity is given in Table I. Practically all of the activity can be assigned to the serum, the difference in the number of enzyme units in blood and serum being due to loss in separation of the cells.

The atropinesterase values of the organs of rabbits containing the enzyme are given in Table II. It was found that the animals without the enzyme in their

sera were also without demonstrable activity in the extracts of any of the organs listed in the table.

In the case of the frog material, three samples of sera, each from the pooled blood of five animals, showed no activity with respect to the splitting of either

TABLE I  
*Atropine Hydrolysis by Defibrinated Rabbit Blood*

Rabbit No.	Atropinesterase units per 0.1 ml. blood		
	Hemolyzed blood	Serum	Hemolyzed red cells
A	55	51	0
B	40	34	0
C	140	110	0

TABLE II  
*Hydrolysis of Atropine by Rabbit Organs and Fluids*

Organ or fluid	Rabbit No.					
	72	82	84*	119	1942	1073
	Atropinesterase values					
Liver.....	72	92	220†			
Intestinal mucosa.....	24	55	117			
Heart auricle.....	10	36	54			
Heart ventricle.....	17	30	41			
Kidney.....	11	33	39			
Spleen.....	6	17				
Adrenal.....	9	12			18	
Lacrimal gland.....	5	27	36			
Gastrocnemius muscle.....	3	7	10			
Brain.....	0	0	6	0		
Aqueous humor.....				0	0	0
Iris.....		17	46		14	
Serum.....	94	124	270	78	77	57

\*Liver infected with coccidiosis.  
†Portion free from infection used.  
Highly infected area had a value of 82.

atropine or homatropine, but the livers of these animals were active on both substrates. The tropinesterase values in a typical experiment, in which the enzyme material was obtained from the pooled livers of five animals, were 70 and 115 for the hydrolysis of atropine and homatropine respectively.

The serum of the guinea pig was also found to exhibit no activity on either atropine or homatropine. The liver actively splits the latter, but has no action

TABLE III  
 Hydrolysis of Tropic Esters

Structure	Name	Enzymatic hydrolysis (tropinesterase units per 0.1 ml. serum)				Corresponding non-enzymatic hydrolysis
		(1 per cent substrate)				
		Horse	Rabbit I	Rabbit II		
$\left[ \begin{array}{c} \text{CH}_3 - \text{CH} - \text{CH}_3 \\   \quad   \quad   \\   \quad \text{NCH}_3 \quad \text{CHOCOCH} \text{---} \text{C}_6\text{H}_{11} \\   \quad   \quad   \\ \text{CH}_3 - \text{CH} - \text{CH}_3 \quad \text{CH}_2\text{OH} \end{array} \right]_2 \text{H}_2\text{SO}_4$	Atropine sulfate (dl-hyoscyamine)	0	96	0	10	
$\begin{array}{c} \text{CH}_3 - \text{CH} - \text{CH}_3 \\   \quad   \quad   \\   \quad \text{H} \quad \text{NCH}_3 \quad \text{CHOCOCH} \text{---} \text{C}_6\text{H}_{11} \\   \quad \text{Br} \quad   \quad   \\ \text{CH}_3 - \text{CH} - \text{CH}_3 \quad \text{CH}_2\text{OH} \end{array}$	l-Hyoscyamine hydrobromide	0	115	0	10	
$\begin{array}{c} \text{CH}_3 - \text{CH} - \text{CH}_3 \\   \quad   \quad   \\   \quad \text{H} \quad \text{NCH}_3 \quad \text{CHOCOCH} \text{---} \text{C}_6\text{H}_{11} \\   \quad \text{Br} \quad   \quad   \\ \text{CH}_3 - \text{CH} - \text{CH}_3 \quad \text{OH} \end{array}$	Homatropine hydrobromide	0	64	0	33	
$\begin{array}{c} \text{CH}_3 - \text{CH} - \text{CH}_3 \\   \quad   \quad   \\   \quad \text{Br} \quad \text{N}(\text{CH}_3)_2 \quad \text{CHOCOCH} \text{---} \text{C}_6\text{H}_{11} \\   \quad   \quad   \\ \text{CH}_3 - \text{CH} - \text{CH}_3 \quad \text{OH} \end{array}$	Novatropine	0	14	0	40	
$\begin{array}{c} \text{CH}_3 - \text{CH} - \text{CHCOOCH}_3 \\   \quad   \quad   \\   \quad \text{H} \quad \text{NCH}_3 \quad \text{CHOCO} \text{---} \text{C}_6\text{H}_{11} \\   \quad \text{Cl} \quad   \quad   \\ \text{CH}_3 - \text{CH} - \text{CH}_3 \end{array}$	Cocaine hydrochloride	0	50	34	148	
$\begin{array}{c} \text{CH}_3 - \text{CH} - \text{CH}_3 \\   \quad   \quad   \\   \quad \text{H} \quad \text{NCH}_3 \quad \text{CHOCO} \text{---} \text{C}_6\text{H}_{11} \\   \quad \text{Cl} \quad   \quad   \\ \text{CH}_3 - \text{CH} - \text{CH}_3 \end{array}$	Tropacocaine hydrochloride	146	11	15	12	
$\begin{array}{c} \text{CH} - \text{CH} - \text{CH}_3 \\ / \quad   \quad   \\ \text{O} \quad \text{H} \quad \text{NCH}_3 \quad \text{CHOCOCH} \text{---} \text{C}_6\text{H}_{11} \\   \quad \text{Br} \quad   \quad   \\ \text{CH} - \text{CH} - \text{CH}_3 \quad \text{CH}_2\text{OH} \end{array}$	Scopolamine hydrobromide	0	72	0	9	
$(\text{CH}_3)_3\text{N}(\text{Cl})\text{CH}_2\text{CH}_2\text{OCOCH}_3$	Acetylcholine chloride*	1510	150	170	130	

\* 0.375 per cent substrate.

on the former as illustrated in Fig. 1. It is noteworthy that the liberation of  $\text{CO}_2$  in the control vessel containing only liver extract in bicarbonate-Ringer



solution was negligible, while that in the substrate control vessels was appreciable, especially during the first 2 hours.

Fresh egg white and yolk were tested separately for atropine-hydrolyzing properties in several instances, and in no case could enzyme action be detected over the experimental period of 360 minutes.

The results of specificity studies are apparent from Tables III and IV.

TABLE IV  
*Comparison of the Hydrolysis of Certain Esters by Rabbit Serum and Cells Expressed in Enzyme Units*

Enzyme material from 0.1 ml. defibrinated blood	Substrate			
	Atropine	Tropacocaine	Cocaine	Acetylcholine
Serum.....	102	20	46	100
Hemolyzed cells.....	0	0	0	125

#### DISCUSSION

It has been known for some time that not all rabbits possess a factor in their blood capable of destroying atropine (2, 3). Metzner and Hedinger (4) found that the weight and age of the rabbit were unrelated to the occurrence of the atropine-destroying factor. Sternberg (5) claimed that the degree of activity of the sera varied with the season, a maximum being reached in December. In the present communication no correlation could be found between the incidence of atropinesterase and any of the animal characteristics observed. Bernheim and Bernheim (6) observed that approximately two out of every three rabbits examined possessed atropinesterase activity, while in the group of sixty-eight rabbits reported in this paper the proportion was more nearly one out of four.

The possibility that the animals devoid of activity possess an inhibiting substance that interferes with the enzyme action has been considered, but it appears unlikely from the fact that a mixture of active and inactive sera contains an activity essentially equal to that of the potent material alone.

It is striking that serum is the richest source of atropinesterase in the rabbit body (Table II). Certain organs such as the lacrymal gland which are unusually rich in cholinesterase (7) contain low concentrations of atropinesterase. The auricle which is about three times as rich as the ventricle in the former enzyme (8), contains approximately the same concentration of the latter. A further difference in the distribution of the two enzymes may be seen from the fact that atropinesterase could not be demonstrated in the blood cells (Table I) while these are rich in cholinesterase (Table IV). The lack of the enzyme in rabbit erythrocytes has been reported by previous investigators employing other methods for the determination of enzyme activity (3, 9, 10).

The absence of atropinesterase in the aqueous humor, and the low activity of iris tissue, would seem to be in accord with the fact that the pupil dilation resulting from dropping atropine into the eye persists for days. In this connection it should be pointed out that since homatropine is effective in the eye for a much shorter period than atropine, one might expect it to be destroyed faster *in vivo*. However, the aqueous humor of a rabbit having no atropinesterase was also found to have no effect on homatropine, and the iris of rabbit No. 1942 (Table II), which had an atropinesterase value of 14 was found to have the same activity on homatropine. It should be kept in mind that the greater non-enzymatic hydrolysis of homatropine (Table III) may be a significant factor.

Since the serum of rabbits is richer in atropinesterase than liver, it is surprising that certain species, such as frog (11-13), guinea pig, rat, cat, and dog (6), have no activity in their serum but do in their liver. Though confirmation of these findings has been obtained in the case of frog, and for guinea pig with respect to serum, it was observed that although liver extract could hydrolyze homatropine, it had no action on atropine (Fig. 1). Bernheim and Bernheim (6) reported liver hydrolysis of both substrates, but their data were not corrected for non-enzymatic substrate hydrolysis. It has been the experience of the authors that occasionally the corrected liver-atropine curve exhibits a small rise which flattens out after 1 or 2 hours and continues in the horizontal. It is extremely doubtful that these initial rises are significant of atropinesterase action since it has been shown that this enzyme is characterized by a linear rise of the hydrolysis-time curve to about 90 per cent of complete scission (1). The hydrolysis of homatropine, but not atropine, by guinea pig liver raises the question of whether there is a homatropinesterase distinct from atropinesterase.

Prior to the work of Bernheim and Bernheim (6), investigations of the enzymatic destruction of atropine were based on the measurement of the disappearance of free atropine rather than on determination of an end-product. Thus it has been claimed that fresh egg white destroys atropine (10, 14), but the disappearance of the drug in the presence of the protein cannot be ascribed to hydrolysis as shown in the present study.

From Table III it is apparent that rabbit serum I is capable of hydrolyzing all of the tropine esters studied. With the exception of cocaine and tropacocaine, it may be seen that both horse serum and rabbit serum II have no effect on these esters. It would appear from the data in the table that *l*-hyoscyamine is hydrolyzed more rapidly than atropine in accordance with previous work (6, 14). However, homatropine, with the hydroxyl group closer to the ester linkage than in atropine, is split more slowly than the latter, while scopolamine is hydrolyzed at an intermediate rate. The last results are not in accord with those of Bernheim and Bernheim (6) who reported that homatro-

pine is hydrolyzed more rapidly than atropine, and scopolamine is usually not attacked. The introduction of another methyl group into the pentavalent nitrogen radical of homatropine results in a marked drop in enzymatic hydrolysis as demonstrated in the case of novatropine.

It has been found by separate control experiments that whether the alkaloid is in the form of the sulfate, bromide, or chloride has no effect on the enzymatic action. The influence of various ions on atropinesterase is being made the subject of another study.

Cocaine is a di-ester in which the tropine portion of the molecule is on the acid side of one ester and on the alcohol side of the other. From a variety of structures previously studied it was found the presence of a nitrogen group in the acid side of an ester prevents enzymatic scission (15, 16). If the analogy applies to the present case one would expect only the benzoyl ester linkage to be attacked, but one cannot be sure that both groups are not subject to enzyme action, especially when it is seen that rabbit serum II can hydrolyze the compound. Since horse serum has no effect, and rabbit serum II contains no atropinesterase, the possibility arises that the cocainesterase in the latter serum may be an enzyme distinct from cholinesterase or atropinesterase.

The small structural variation between tropacocaine and atropine or homatropine exerts a profound effect upon the enzyme specificity. All three of the sera employed hydrolyze tropacocaine, and by comparison with the rates of hydrolysis of acetylcholine it would appear that cholinesterase might be the active agent in the tropacocaine scission. A test of this possibility was made based on the fact that the red cells of rabbit blood contain cholinesterase even though they are devoid of atropinesterase. Hence both the serum and cells should hydrolyze tropacocaine if cholinesterase is capable of acting upon the substance. From Table IV it is apparent that cholinesterase is without action on tropacocaine. Therefore the possibility is presented that a tropacocainesterase may also exist as an enzyme distinct from cholinesterase, atropinesterase, and cocainesterase.

The great difference between the non-enzymatic hydrolysis of cocaine and tropacocaine must be ascribed to the presence of the additional methyl ester group in the former. This group probably undergoes scission with ease.

#### SUMMARY

Atropinesterase was found to exist in approximately one out of every four rabbits, and no relation could be observed between the incidence of the enzyme and season, sex, color, age, or weight. The occurrence of the enzyme was also shown to be unrelated to that of cholinesterase.

The distribution of atropinesterase in the blood and organs of rabbits was studied; the animals devoid of the enzyme in their blood contained no demonstrable activity in any of the organ extracts tested.

The presence of atropinesterase in frog liver, and its absence from the serum, has been confirmed. Hydrolysis of homatropine, but not atropine, by guinea pig liver was observed, while the serum was without action on either of the compounds. On this basis the possibility arises that guinea pig liver contains a homatropinesterase enzyme separate from atropinesterase.

It was shown that lack of atropinesterase activity in certain rabbits is not likely to be due to the presence of a naturally occurring inhibitor.

It has been demonstrated that contrary to previous indications neither fresh egg white nor yolk possess atropinesterase activity.

The specificity of tropinesterases was investigated and evidence was presented for the possible existence of two distinct enzymes, cocainesterase and tropacocainesterase.

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# THE INFLUENCE OF PROTEINS ON THE REACTIVATION OF YEAST INVERTASE

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Saul and Nelson (1) studied the influence of proteins on the activity of yeast invertase, and found that native and denatured proteins accelerate the activity of purified invertase preparations at pH 3, but not at pH 4.5, the pH optimum of the enzyme. Herriott (2) had shown that yeast invertase inactivated by acid can be reactivated by following Anson and Mirsky's (3) procedure of allowing a solution of the inactivated enzyme to stand for varying intervals of time at pH 6.

In general, Herriott was able to obtain a greater per cent reactivation using fairly crude invertase preparations. However, Saul and Nelson (1) found that only highly purified invertase preparations respond to the accelerative influence of added proteins. These authors suggest therefore, that crude preparations contain an excess of protein so that the addition of more protein seems to be without further effect. The possibility exists that there may be a relation between the reactivation of invertase preparations containing a relatively large amount of extraneous protein and the accelerative effect on the activity of highly purified preparations due to added proteins. It is the purpose of this investigation to test this possibility by studying the effect of added proteins on the degree of reactivation of acid-inactivated yeast invertase.

The procedures used in this study were in general similar to those used by Herriott (2) with minor modifications. The activities of the invertase solutions were estimated by the initial rates of hydrolysis of 16 per cent sucrose solutions at pH 4.5 and 25°C., except where specifically stated otherwise.

The various invertase preparations used were diluted to give an initial velocity of sucrose inversion of around 2.0–2.5° per minute. It was then demonstrated for each different enzyme preparation used that when brought to a pH below 1.2 and kept at this pH for 3 minutes, followed by adding alkali to a pH of 4.5 and allowing the solution to stand for 45 minutes, that the enzyme was completely inactive. However, when acid-inactivated invertase solutions were brought to pH 6, and allowed to stand for 45 minutes, reactivation occurred in complete agreement with Herriott's findings.

For each preparation used in the present investigation the pH reactivation optimum

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\* This work was begun in the laboratory of Professor J. M. Nelson of Columbia University and continued with his approval.

was found (these varying between pH 5.8 and 7.0) and the experimental conditions were so arranged as to have the reactivation occurring at this pH.

The following is a typical experiment on the influence of proteins on the reactivation of invertase. In each case two experiments are carried out; one serving as a control, and differing from the other only in that no protein is added.

To 25 ml. of invertase in 0.015 M phosphate-citrate buffer at pH 4.5 (initial velocity of hydrolysis 2.0–2.5° per minute) is added 1 ml. of a water solution containing 10 mg. of egg albumin. To this is added 10 ml. of 0.428 N HCl, and 10 ml. of the resulting mixture are removed for a pH determination (pH equals 1.10). The mixture is kept at this pH for 30 minutes. At the end of this time 10 ml. of 0.327 N NaOH are added bringing the pH to 5.89. This mixture is allowed to reactivate for 45 minutes, and then 25 ml. are removed and added to 100 ml. of 20 per cent sucrose solution buffered with 0.03 M phosphate-citrate buffer, so that the final pH of the hydrolysis mixture is 4.5. (The remaining 10 ml. of reactivated invertase are used to determine the pH at which reactivation took place.)

The initial course of the hydrolysis is now followed polarimetrically in the usual manner, five samples being obtained for each hydrolysis. The control is then treated in exactly the same way, except that 1 ml. water is added at the start, the pH of inactivation being 1.09 and the pH of reactivation being 5.86 in this experiment.

The results of this experiment were as follows:

(A) Reactivation in presence of 10 mg. of egg albumin			
Hydrolysis sample	$\Delta$ Time	$\Delta$ Rotation (in degrees)	$\Delta R/\Delta T$
	<i>min.</i>		
1	0	0	—
2	4	0.52	0.130
3	8	1.06	0.133
4	12	1.58	0.132
5	16	2.16	0.135
Mean rate = 0.133° per minute			
(B) Control reactivation			
1	0	0	—
2	4	1.09	0.273
3	8	2.16	0.270
4	12	3.19	0.266
5	16	4.39	0.274
Mean rate = 0.271° per minute			

Per cent inhibition of reactivation due to added protein =

$$\frac{(\text{Rate of control experiment} - \text{rate of experiment with added protein}) \times 100}{\text{Rate of control}}$$

$$= \frac{(\text{here}) 0.271 - 0.133}{0.271} \times 100 = 51 \text{ per cent}$$

Since all the variables such as time, temperature, and pH, have been held constant, it is possible to attribute the difference in extent of reactivation to the added protein.

A series of experiments of this nature were carried out, with the various proteins being added at one of three possible points, *i.e.* at the start (invertase at pH 4.5), immediately after bringing the invertase solution to the inactivation pH of 1.1, or immediately after bringing the enzyme to the reactivation pH of about 6. Since it was found that the results on adding the proteins at pH 4.5 and at pH 1.1 were the same, Table I lists the effect of proteins at two general points of addition; namely at pH 4.5 or less than 1.2, and at pH 6.

The proteins used in this study were highly purified preparations, many of which were obtained from various workers whose names are listed at the end of the paper. During the early stages of this work highly purified invertase solutions were used, which were prepared by Mr. Stanley Lewis of Columbia University. It was found that results using highly purified yeast invertase were the same as those obtained with the cruder commercial preparation known as "Convertit" which is a product of Wallerstein Co. of N.Y. Accordingly, "Convertit" was used in the later stages of the investigation.

Examination of Table I reveals some interesting facts. Under no conditions was an increase in reactivation obtained. Secondly, all but three of the sixteen proteins tested inhibited the reactivation of invertase to some extent when added at pH 4.5 or 1.1, the three exceptions being carbohydrate-free horse crystalalbumin, gelatin, and lactoglobulin, which also did not inhibit at the reactivation pH. Only five proteins inhibited the reactivation of invertase, when added at pH 6. Of these, two: trypsin and chymotrypsin are proteolytic enzymes which are active at pH 6, and three, donkey globin, ox globin, and serum albumin are non-enzymatic. Another point to be noticed is that a general distinction in quantitative effect can be drawn between the proteolytic enzymes and the non-enzymatic proteins which were added. The former when present in amounts as small as 0.1 mg. caused an inhibition of 70–100 per cent, while the latter when present in far larger quantities (10 mg.) gave inhibitions varying from 14–87 per cent. For comparison it might also be added that as much as 0.5 mg. tobacco seed globulin resulted in a zero inhibition.

The inhibition of the reactivation on the part of the proteolytic enzymes may, perhaps, be considered a function of their enzymatic activity, and, indeed, several facts appear to support this possibility.

Thus, in one experiment 0.06 mg. pepsin which had been inactivated by keeping at pH 10.5 for half an hour (6) (proteolytic activity proven absent by the method of Anson (7)) was added to the invertase solution at pH 1.1. In the native state, this quantity of pepsin was sufficient to cause 100 per cent inhibition of reactivation. In its denatured form, however, it gave 0 per cent inhibition. Also, 5 mg. of chymotrypsinogen added to an invertase solution immediately after bringing it to pH 1.1 led to 14 per cent inhibition of reactivation.



tion of invertase. 5 mg. of chymotrypsin, on the other hand, added under the same conditions, caused an inhibition of reactivation of 100 per cent. This experiment is probably more satisfactory in demonstrating the correlation of

TABLE I  
*Effect of Proteins on Reactivation of Invertase*

Protein	Quantity added	Inhibition when added at pH 4.5 or 1.1	Inhibition when added at pH 6
	mg. dissolved	per cent	per cent
1a Egg albumin*	10	51	0
1b " "	12	45.3	0
2. Carbohydrate-free horse crystalbumin (4)	10	0	0
3a Edestin	10	74	0.4
3b " "	10	73	0
4. Gelatin	10	0	0.5
5. Donkey globin	10	41	37
6. Ox globin	10	87	87
7. Lacto-globulin	10	0	0
8. Phosphorylated serum albumin	12	16	—
9. Pseudoglobulin $\gamma$ (5)	10	61	0
10. Serum albumin	12	36	36
11. Tobacco seed globulin	10	75	0
	5	66	—
	3	45.3	—
	1	15.7	—
	0.5	0	—
12. Chymotrypsin	5	—	100
	2	98	—
	0.1	85	—
13. Chymotrypsinogen	5	14	2
14. Pepsin	>0.06	100	0
	0.06	100	—
	0.03	93	—
	0.01	70	—
	0.001	65	—
15. Pepsin denatured at pH 10.5	0.06	0	—
16. Trypsin	0.10	70	—
	10	100	100

\* a and b refer to different preparations of the same protein.

the inhibitory effect and proteolytic activity than that of denaturing the pepsin, since although chymotrypsinogen has but 1/10,000 the proteolytic activity of chymotrypsin (probably due to traces of chymotrypsin) it is in other properties quite similar to chymotrypsin, according to Kunitz and Northrop (8).

Finally, a third line of evidence to support the idea that the inhibition by the proteolytic enzymes mentioned may be associated with their enzymatic activity

is indicated by the following experiments. Pepsin when added to invertase at pH 1.1 inhibits the reactivation 100 per cent. However, when pepsin is added to invertase immediately after bringing the invertase to pH 6 (at which pH the pepsin is proteolytically inactive), there is no inhibition of reactivation whatever.

Concerning the inhibition by the non-enzymatic proteins listed in Table I, the authors do not offer any suggestions to account for the phenomenon. The possibility, however, remains that the inhibition by the various non-enzymatic proteins may be due to their contamination with proteolytically active substance. The presence of 0.005 mg. of pepsin in 10 mg. of protein would be sufficient to cause 50 per cent inhibition of reactivation. The method of Anson (4) (modified to 1 hour's digestion to insure sensitivity to as little as 0.005 mg. pepsin) was used to investigate this possibility. All the proteins mentioned in

TABLE II  
*Effect of Trypsin and Chymotrypsin on Native Invertase*

Enzyme	Quantity	pH	Time of contact of native invertase and enzyme	Velocity of invertase before addition of proteolytic enzyme	Velocity after contact for time shown
	mg.				
Trypsin	10	4.6	68 min.	0.0103 °/min.	0.0108 °/min.
"	10	6.75	61 "	0.0301 "	0.0301 "
"	13	7.06	66½ hrs.	0.080 "	0.080 "
"	10	7.0	45 "	0.094 "	0.094 "
Chymotrypsin	10	6.3	67 "	0.532 "	0.531 "

Table I were tested for peptic activity, and in addition, serum albumin and globin were tested for tryptic activity since the latter inhibited invertase reactivation when added at pH 6. All were found to be proteolytically inactive.

The question now arises; do the proteolytic enzymes affect native invertase? There appears to be some disagreement in the literature on this point (1, 9).

The possibility was tested in two ways. In the first method, the native invertase was allowed to stand in contact with the proteolytic enzyme at different pH's for various lengths of time. The results, as indicated in Table II, show the native invertase to be unaffected by the presence of trypsin and chymotrypsin. Another type of experiment used to obtain information on this question was as follows:

10 mg. of trypsin were added to invertase which had reactivated at pH 6.2 for 1 hour. The invertase was now allowed to stand at pH 6.2 for a total of 5 hours, and its activity then determined. One control was allowed to reactivate at pH 6.2 only 1 hour and its activity immediately obtained. Another control was allowed to reactivate at pH 6.2 for 5 hours before testing its activity. Results: Velocity of invertase to which 10 mg. of trypsin were added after 1 hour (5 hours total standing) = 0.0134

degree per minute. Velocity of control reactivated for 1 hour = 0.0133 degree per minute. Velocity of control reactivated for 5 hours = 0.0306 degree per minute.

Other experiments of this type gave similar results and led to the following conclusions: (1) Trypsin instantaneously acts to prevent further reactivation of inactive invertase, (2) trypsin does not affect whatever invertase has already reverted to the active form in the reactivation process.

It was also considered interesting to determine whether reactivated invertase would exhibit an effect typical of the original native invertase, namely the effect described by Saul and Nelson (1). For this experiment it is necessary that a highly purified yeast invertase preparation be used.

A highly purified yeast invertase was inactivated at pH 1.1, as usual, and then brought to pH 6.2 and allowed to reactivate at this pH for 1 hour. The solution was then carefully brought to pH 3.0 with dilute HCl. The solution was now divided into two parts, and to one part 10 mg. of trypsin were added, and the activity of this and the control were compared in buffer at pH 3.0.

This experiment was also repeated using 6 mg. gelatin, instead of trypsin, as the foreign protein added.

Increases of 19 per cent and 25 per cent respectively at pH 3.0 were obtained in these experiments due to added protein, and are in accord with findings reported by Saul and Nelson (1) for native invertase.

It will be remembered that edestin and tobacco seed globulin did not inhibit the reactivation of invertase when these proteins were added to acid-inactivated invertase which had just been brought to pH 6.2, but did when added at pH 4.5 or 1.1. The following experiment was carried out in an attempt to obtain some further information concerning the inhibitory effect using these proteins.

Edestin, tobacco seed globulin, and pepsin were separately brought to pH 1.1 and allowed to remain at that pH for one-half hour. Then 10 mg. edestin, 10 mg. tobacco seed globulin, and 1 mg. pepsin were each added to separate portions of acid-inactivated invertase which had just been brought to pH 6. The velocities were then compared with a control and the inhibitions obtained were 65 per cent with the tobacco seed globulin, 35 per cent with edestin, and 0 per cent with 1 mg. pepsin.

This experiment suggests that in the case of edestin and tobacco seed globulin the inhibitions described in Table I may be ascribed to some special changes in the edestin and tobacco seed globulin when these are brought to pH 1.1 and the subsequent interaction of these specially changed proteins with inactive invertase at pH 6.2 thereby preventing its normal regeneration.

The pepsin experiment points to the previous idea that in this case the effect is proteolytic since 1 mg. pepsin is sufficient when added at pH 4.5 or 1.1 in the usual way to prevent any reactivation, while under these last conditions no inhibition at all was obtained.

## SUMMARY

1. Acid-inactivated yeast invertase could not be regenerated in the presence of the proteolytic enzymes trypsin, pepsin, and chymotrypsin.
2. Certain foreign proteins of non-enzymatic nature partially inhibited the reactivation of acid-inactivated invertase.
3. Certain proteins as gelatin, lacto-globulin, and carbohydrate-free horse crystalbumin did not prevent the reactivation of invertase at all.
4. Highly purified reactivated invertase was shown to exhibit an effect typical of original native invertase; that is, acceleration of its activity in presence of foreign protein at pH 3.0.
5. Native invertase was not digested by trypsin and chymotrypsin.
6. The addition of trypsin and chymotrypsin to reactivating invertase did not affect the invertase which had already reverted to the active form, but prevented further reactivation of inactive invertase.

We are greatly indebted to the following for their kindness in supplying us with proteins used in this work: Dr. Max Bergmann, for lactoglobulin, Dr. Henry Borsook, for edestin, and egg albumin (three times recrystallized), Dr. Edwin J. Cohn, *et al.*, carbohydrate-free horse crystalbumin, and pseudoglobulin- $\gamma$ , Dr. Michael Heidelberger, Dr. Henry P. Treffers, and Mr. Manfred Mayer, for serum albumin, phosphorylated serum albumin, and egg albumin, Dr. Roger M. Herriott, for pepsin (twice recrystallized), trypsin (three times recrystallized), chymotrypsin (three times recrystallized), chymotrypsinogen (seven times recrystallized), Dr. Abraham Mazur, tobacco seed globulin, edestin, and gelatin, and Dr. Alfred E. Mirsky for donkey globin.

We also wish to thank Dr. A. E. Mirsky for discussing this work with us and making helpful suggestions.

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# OSMOTIC PROPERTIES OF THE EGG CELLS OF THE OYSTER (OSTREA VIRGINICA)

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There is abundant evidence that living cells function as osmotic systems (1). Quantitative studies, however, on osmotic equilibria and on the kinetics of osmosis with animal cells have been hindered by lack of suitable material. To serve as osmometers for experimental purposes, cells should meet the following requirements: 1. They should be obtainable as isolated cells of a single type. Cell aggregates, such as tissues or organs are unsatisfactory, since they contain not only cells of more than one type, but also blood vessels, lymphatics, tissue spaces, and intercellular substances—all having different properties. 2. The cells should be free to undergo changes of volume, since osmotic phenomena involve such changes. In tissues, free changes of cell volume are interfered with by mutual pressure of component parts; even certain isolated cells are unsuitable, because of the rigidity of their surface membranes (2). The size and shape of the cells should be such as to permit accurate measurement. Few types of animal cells are known that fulfill these requirements, wherefore quantitative investigations of osmotic phenomena, and of the related property of permeability, have been confined almost entirely to two types of cells: mammalian erythrocytes and echinoderm egg cells. In the course of search for new material, the eggs of two other forms of marine invertebrates which belong to different phyla—the annelid *Chaetopterus pergamentaceus* and the mollusc *Cumingia telenoides*—have also proved well adapted for osmotic studies (2). Unfortunately, neither of these forms were readily available, nor—as the number of eggs from any single specimen of *Cumingia* sufficient for some experiments.

Further attempts to find suitable cells disclosed that the unfertilized egg of the oyster is excellent material. This animal is widely distributed, its eggs are very abundant, and are obtainable without difficulty. To stimulate shedding of eggs, oysters are placed in bowls of sea water, the temperature of which is suddenly raised to 32–35°C., after which the water is allowed to cool.<sup>1</sup> When

<sup>1</sup> Eggs thus obtained are entirely uninjured and may be fertilized. The mechanism of the spawning of oysters has been extensively studied by Dr. Paul S. Galtsoff, of

first shed, the eggs are usually ovoid or pear-shaped, but after repeated washing in sea water they become, in a high percentage of samples, uniformly spherical (Fig. 1); the diameter was found to range (in samples from 10 animals) from  $50.8$  to  $54.4\mu$ , with  $52.2\mu$  the average. Compared to other marine eggs, the egg of the oyster is relatively small.<sup>2</sup>

For measuring the eggs a diffraction method was used, the details and advantages of which have been described in previous communications (2, 4). Each measurement of the diffraction pattern gives a statistical average of many thousands of individual cells. Accurate measurements can be made at intervals of 5 seconds (and under favorable conditions in less time); this is a particular advantage when, as in oyster eggs, osmotic volume changes are rapid.



FIG. 1. Egg cells of *Ostrea*. The photograph shows the eggs as isolated cells of spherical shape and of uniform size.

The present experiments deal with the question "How good a natural osmometer is the unfertilized egg of the oyster?" To answer this question we have studied: the equilibria the cells attain in several different concentrations of sea water, the reversibility of the volume changes, and the course of osmotic swelling and shrinking. From the data obtained, the permeability of the cells to water and to two solutes, diethylene glycol and glycerol has been computed. The results of these experiments will now be taken up in order.

the U. S. Bureau of Fisheries. He states (3) that "the temperature (such as the above) to which the oysters were subjected in the laboratory lies within the thermal range of their normal habitat and the conditions of the experiment cannot be considered abnormal or even unusual." There are other methods for inducing spawning, but they involve the use of sperm or of chemical agents which we wished to avoid. We are greatly indebted to Dr. Galtsoff, and to his assistant, Mr. B. Böving, for furnishing us most of the cell suspensions used in the present experiments.

<sup>2</sup> For example, *Chaetopterus*,  $100\mu$ ; *Arbacia*  $75\mu$ , *Cumingia*  $65\mu$ , approximately.

### 1. Applicability of the Law of Boyle-van't Hoff. Volume of Osmotically Inactive Material

If cells were perfect osmometers, their volume should increase in direct proportion as the osmotic pressure of the medium with which they are in equilibrium decreases; stated alternatively, the product of volume and pressure should be constant. It has been shown (1) for several types of living cells

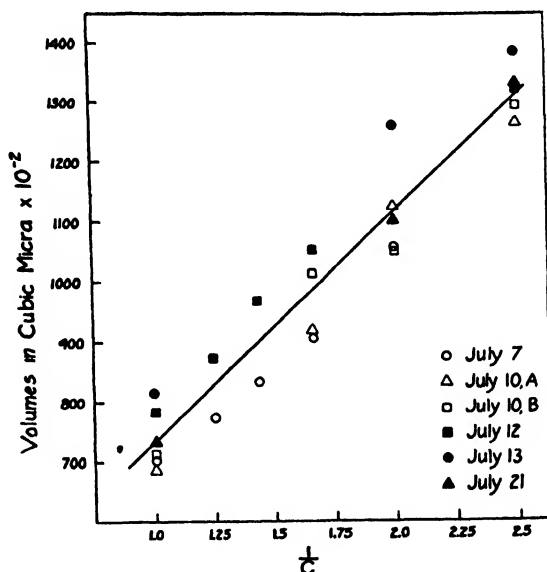


FIG. 2. Applicability of Boyle's law to oyster eggs. Results of six equilibrium experiments. Volumes of cells are plotted against reciprocals of corresponding concentrations of sea water with which they are in equilibrium (concentration of ordinary sea water = 1.0). The graph drawn weighs all the observed points and is linear; it represents the equation  $P(V - b) = \text{constant}$ . The volume of osmotically inactive cell contents,  $b$ , is obtained by extrapolation of the graph to  $\frac{1}{C} = 0$  (this extrapolation is not shown in the figure). The mean value of  $b$  is 44 per cent of the initial cell volume.

(plant cells, erythrocytes, marine eggs) that this relation holds provided that cell volume is corrected for osmotically inactive cell content. The significant volume is not the volume of the cell as measured, but is this volume diminished by a quantity which represents the space occupied by osmotically inactive material. Boyle's law as applied to cells becomes:

$$P(V - b) = K \text{ constant} \quad (1)$$

where  $P$  is the osmotic pressure of the dissolved substances in the cell (this pressure at equilibrium is identical with the known osmotic pressure of the



medium),  $V$  is the measured cell volume, and  $b$  the space within the cell occupied by osmotically inactive material (this term includes bound water if any exist).

The applicability of Boyle's law was tested in duplicate or triplicate experiments with eggs from six oysters. The cells were measured in ordinary sea water, and in several dilutions of sea water with which they had been brought to equilibrium. The results are shown in Fig. 2, in which the observed volumes are plotted against the reciprocals of the corresponding concentrations of sea water; a straight line may be fitted to the points, which is in accordance with equation 1. Thus it is evident that the egg cells of the oyster closely obey the law of Boyle-van't Hoff, provided that the cell volume is corrected for osmotically inactive contents.

The correction factor,  $b$ , may be computed from the observed volumes arithmetically, or, more simply by extrapolating the linear graph of Fig. 2; the intercept with the axis  $\frac{1}{C} = 0$  gives directly the mean volume of osmotically inactive cell content. The value of  $b$  computed in this way<sup>3</sup> is equal to 44 per cent of the cell volume in ordinary sea water. The amount of  $b$  for the oyster egg is much greater than  $b$  for the *Arbacia* egg which has only 12 per cent of dead space (4, 5); on the other hand, the amount for the oyster egg is approximately the same as  $b$  for mammalian erythrocytes. In different plant cells, even greater variations of  $b$  values are found; for certain yeasts the correction factor is 64 per cent of cell volume; on the other hand, for certain other cells which have large sap vacuoles and an extremely thin layer of protoplasm,  $b$  approaches zero (1).

## 2. Reversibility of Osmotic Swelling; Preservation of Semipermeability

In the experiments discussed in the preceding section, the relation of cell volume to osmotic pressure was investigated over a wide range of concentration of sea water—from 100 to 40 per cent, corresponding to pressures of 22 to 8.8 atmospheres. At all concentrations, the volume of the cells reached a steady state, which for a number of hours remained unaltered. This fact made it unlikely that swelling in the hypotonic solutions had led to injury and consequently to loss of semipermeability.

Reversibility of the volume changes was now tested by returning the swollen cells to ordinary sea water, and measuring them after they had again come into equilibrium. The results of a representative experiment, with duplicate meas-

<sup>3</sup> A graphic method for evaluating  $b$  is perhaps preferable to arithmetic computation, for such computations are greatly affected by slight accidental errors of measurement, whereas a graphic method at once weighs all the points. The values of  $b$  obtained arithmetically ranged from 31 to 56 per cent, with two-thirds of the values being between 40 and 48 per cent.

urements for each concentration, are shown in Table I. It is seen that the original and final volumes are in good agreement. It may consequently be concluded that osmotic volume changes of oyster eggs (within the range stated) are reversible, and that the semipermeability of these cells remains intact.

### 3. Kinetics of Osmosis

The rates at which the oyster eggs attain osmotic equilibria were now studied as follows. After measuring their initial size, the cells were mixed with diluted sea water in such proportion as to give a suspension of suitable density of cells in a medium of known concentration. This suspension was quickly introduced into the chamber of the apparatus and the changes in

TABLE I

Reversibility of volume changes. In this experiment, performed in duplicate, at 22°C., cells were measured in ordinary sea water (column 1), then swollen in 40 and 50 per cent sea water, respectively, and remeasured after 1 hour (column 2 and 3). They were then returned to ordinary sea water and again measured after 1 hour (last 2 columns). (The cells reached equilibrium within a few minutes after transfer to the hypotonic solution, or after returning them from such solutions to ordinary sea water.) For ease of comparison diameters, as well as volumes, are given. It is seen that the cells return approximately to their original size.

Initial size in 100 per cent sea water	Size in equilibrium with		Final volume	
	100 → 40	100 → 50	40 → 100	50 → 100
72,780 $\mu^3$ (51.8) $\mu$	134,100 (63.5)	110,300 (59.5)	73,200 (51.9)	71,520 (51.5)
73,620 $\mu^3$ (52.0) $\mu$	132,200 (63.2)	110,300 (59.5)	73,620 (52.0)	71,100 (51.4)

size of the cells were followed by making readings of the diffraction pattern until a steady state was reached.

The course of swelling and of shrinking during endosmosis and exosmosis, respectively, is illustrated in Figs. 3 and 4. It is seen that either swelling or shrinking is completed in considerably less than 5 minutes. Osmotic changes of oyster eggs are rapid when compared to similar processes in *Arbacia* eggs, which require approximately 30 minutes to reach equilibrium.

With data obtained from the measurements of oyster eggs made during swelling or shrinking in anisotonic solutions we can test the applicability of equations (based upon theoretical grounds) which for other types of living cells have satisfactorily described the course of osmotic volume changes. It has been shown (4,6) that rate of change of cell volume is, at any instant, proportional to the area of the surface and to the difference in osmotic pressure existing between the cell and the surrounding medium; i.e.,

$$\frac{dV}{dt} = K \cdot S \cdot (P - P_0) \quad (2)$$

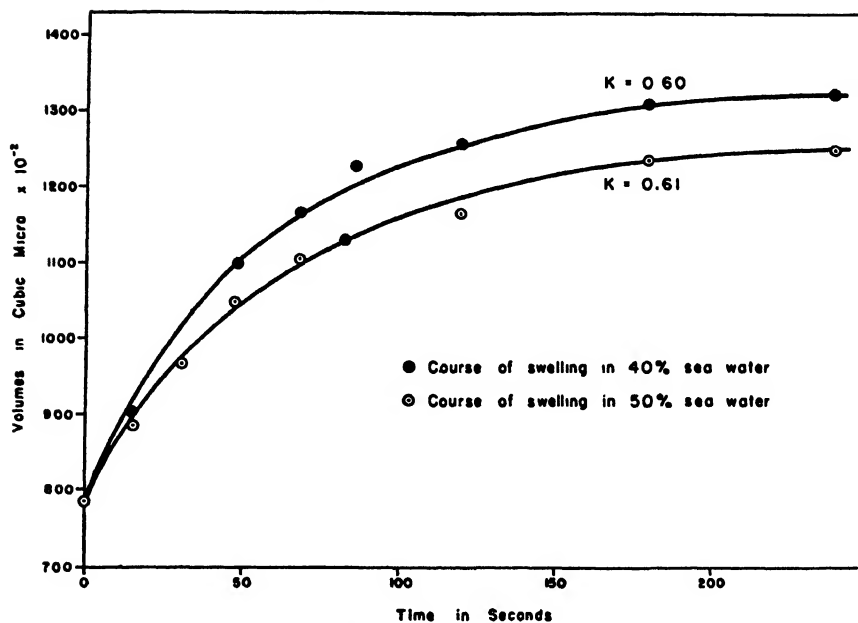


FIG. 3. Course of swelling of cells in two concentrations (40 and 50 per cent) of hypotonic sea water. Permeability constants ( $K$ ) are in  $\mu^3/\text{min.}/\mu^2/\text{atmosphere}$ .

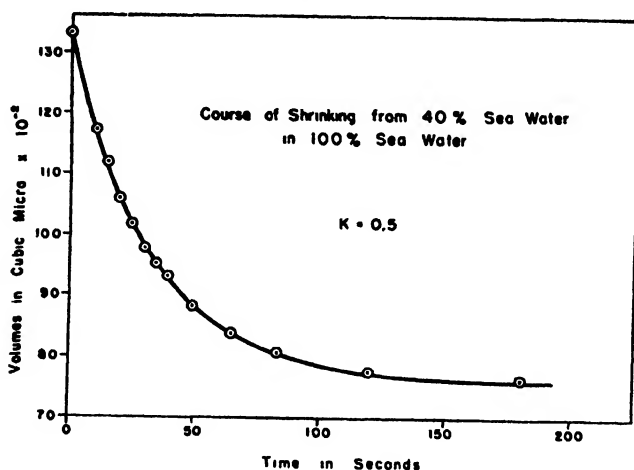


FIG. 4. Course of shrinking of cells upon transfer from 40 per cent sea water (with which they had previously been brought to equilibrium) to ordinary sea water.

where  $\frac{dV}{dt}$  is rate of change of cell volume due to passage of water, either inward or outward;  $S$  is the area of the cell surface,  $P$  the osmotic pressure of the in-

terior of the cell,  $P_0$  that of the surrounding medium, and  $K$  is a factor of proportionality. Correcting cell volume for  $b$  (equation 1), and making necessary substitutions before integrating, equation 2 when integrated becomes:<sup>4</sup>

$$k(36\pi)^{\frac{1}{2}} \cdot P_0(V_0 - b)t = (V_0 - b) \left[ \left( 1 - \frac{b}{V_0} \right) \cdot V_0^{\frac{1}{2}} \left( \frac{1}{2} \ln \frac{V_0 - V}{(V_0^{\frac{1}{2}} - V^{\frac{1}{2}})^2} + \sqrt{3} \tan^{-1} \frac{2V^{\frac{1}{2}} + V_0^{\frac{1}{2}}}{\sqrt{3} V_0^{\frac{1}{2}}} \right) - 3V^{\frac{1}{2}} \right] V_{t=0} \quad (3)$$

In the application of this equation to the experimental data it will be convenient to designate the entire right hand side of equation 3 as  $f(V, V_0)$ . Using the data of two representative swelling experiments and plotting  $\frac{1}{(36\pi)^{\frac{1}{2}} \cdot P_0(V_0 - b)} \cdot f(V, V_0)$  against time, the fit obtained is linear (Fig. 5). This is in harmony with the demands of the equation, which may therefore be regarded as describing satisfactorily<sup>5</sup> the course of osmotic volume changes of the oyster egg.

#### 4. Permeability of the Oyster Egg to Water during Endosmosis and Exosmosis

The permeability of a cell to water,  $K$  of equations 2 and 3, is defined as the amount of water that enters or leaves the cell in unit time, through unit of cell surface, as a result of a unit of pressure. In convenient units, permeability is expressed as the number of cubic micra of water that pass per minute through each square micron of cell surface, per atmosphere of difference in osmotic pressure between the cell interior and the medium. This quantity may be obtained directly from the slopes of linear graphs such as are shown in Fig. 5.

<sup>4</sup> The equation as here given is a somewhat simplified form of that previously published (6). The expression  $\frac{V_0 - V}{(V_0^{\frac{1}{2}} - V^{\frac{1}{2}})^2}$  in the present equation has been obtained

by multiplying numerator and denominator of  $\frac{V_0^{\frac{1}{2}} + (V_0 V)^{\frac{1}{2}} + V^{\frac{1}{2}}}{(V_0^{\frac{1}{2}} - V^{\frac{1}{2}})^2}$  in the old form by  $V_0^{\frac{1}{2}} - V^{\frac{1}{2}}$ ; this eliminates calculation of the three quantities  $V_0^{\frac{1}{2}}$ ,  $V^{\frac{1}{2}}$ , and  $(V_0 V)^{\frac{1}{2}}$ , none of which appears elsewhere in the equation. We are obliged to Dr. T. N. Harris, of the Laboratory of Bacteriology, University of Pennsylvania, for drawing our attention to this simplification.

The symbol  $V_0$  indicates volume of the cell at equilibrium,  $V_0$  and  $P_0$  are volume and pressure, respectively, in ordinary sea water. Suggestions for the construction of conversion charts, by means of which the computation is rendered a relatively easy task, are given on page 409 of a previous publication (6).

<sup>5</sup> More correctly this equation describes the swelling and shrinking process from the beginning to approaching equilibrium. As the cells approach their equilibrium, deviations from equation 3 appear which are as yet unexplained.

In these experiments, the values of  $K$  for water entering cells were the same, namely 0.6. In similar experiments with eggs from four other oysters which were caused to swell in either 40 or 50 per cent sea water, permeability ranged from 0.5 to 0.6, with most values close to an average permeability of 0.6 for endosmosis.

For the reverse process, exosmosis, permeability may be computed by two methods. In the first method, equation 3 is used and the value of perme-

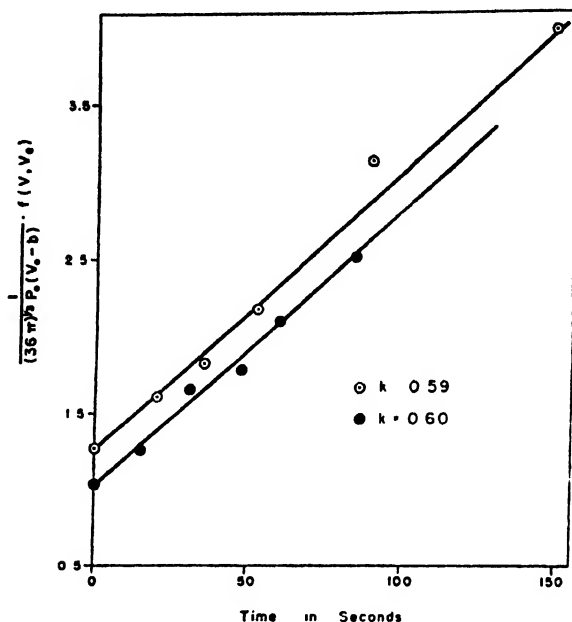


FIG. 5. Applicability of equation 3 to swelling of oyster eggs in 50 per cent sea water.

Results of experiments with cells from two animals. Plotting  $\frac{1}{(36\pi)^{\frac{1}{2}} P_0 (V_0 - b)} \cdot f(V, V_0)$  against time yields linear graphs, showing that the equation fits the data. The slopes of the lines are the values of the permeability factor,  $K$ .

ability derived as has been described for endosmosis. In three duplicate experiments, cells previously swollen in 40 or 50 per cent sea water were measured during shrinking after their return to ordinary sea water; in these experiments the values of permeability for exosmosis averaged 0.5.

By a second method, based upon entirely different procedures, values of permeability were slightly, but not significantly, higher. This method, developed by Jacobs (7) makes it possible to evaluate permeability to water and to solutes as well. The cells are measured in ordinary sea water, and then transferred to a medium made by dissolving in sea water 0.5  $\mu$  of a relatively harmless substance to which the cells are permeable—in the present experi-

ments, diethylene glycol or glycerol. In this initially hypertonic medium the cells at first shrink, but as the solute penetrates, a minimum volume is attained and then the water lost during shrinking is gradually regained (Fig. 6). From

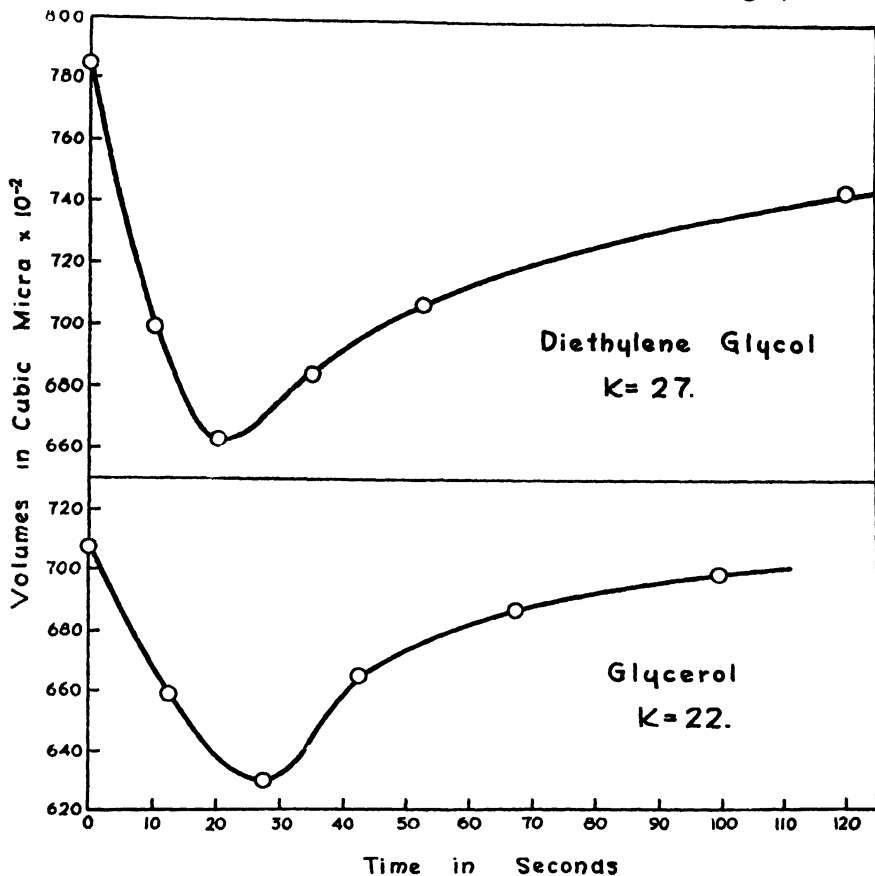


FIG. 6. Two experiments with eggs from different oysters showing the course of shrinkage and subsequent swelling when placed in a solution of 0.5 M diethylene glycol (upper graph) or glycerol (lower graph) in sea water. Volumes correspond to averages of diffraction scale measurements. Permeability constants for solutes are in mols  $\times 10^{-15}$ /min./ $\mu^2$ /mol per liter.

the initial size, minimum value obtained, and time of attaining this minimum it is possible to compute both the permeability to the solute and the water.<sup>6</sup>

<sup>6</sup> For details of computation see (7). In the determinations of the permeability constants for water by this method, the volume of osmotically inactive cell contents has not been considered. Probably no serious error is thereby introduced for such correction is of less importance when dealing with volumes which differ only slightly from the initial size; i.e., less than 15 per cent in the present experiments.

The significant data of five experiments with diethylene glycol as the penetrating solute are recorded in Table II; in the last column are stated the values of permeability to water; the average is 0.7. In three similar experiments with glycerol, the average is 0.6. These values are but slightly higher than the values computed from the course of shrinking by the first method.

By two entirely different methods, therefore, the permeability of the oyster egg is found to be 0.5–0.7 for exosmosis. For endosmosis, as shown above, the values obtained are exactly the same. This agreement is of considerable interest, since with another type of living cell, the *Arbacia* egg, water appears to leave the cell more readily than it enters: the calculated value of permeability for exosmosis is at least 25 per cent greater than the value of endosmosis.<sup>7</sup> No

TABLE II

Permeability of eggs of *Ostrea* to water, and to diethylene glycol, at 22°C. Permeability values are given in the last two vertical columns. Permeability to water is expressed as number of cubic micra of water which enter the cell, per minute, through each square micron of cell surface, per atmosphere of difference in osmotic pressure. The values for diethylene glycol are the number of mols  $\times 10^{-18}$  of this substance which pass per minute through each square micron of cell surface, at a concentration difference of 1 mol per liter.

Experiment	Initial volume	Minimum volume	Time to minimum volume	$\frac{V_m}{V_0}$	Permeability to diethylene glycol $\times 10^{-18}$	Permeability to water
			min.			
1	68,600	60,100	0.38	0.875	29.6	0.73
2	78,390	66,240	0.33	0.845	26.6	0.84
3	77,950	69,500	0.42	0.892	28.2	0.64
4	73,200	61,600	0.38	0.845	24.2	0.81
5	70,690	63,770	0.50	0.902	27.3	0.50
Mean.....	73,770	64,240	0.40	0.872	27.2	0.70

such difference is found for the oyster egg, which in this respect may be regarded as a more perfect osmotic system.

#### 5. Permeability to Diethylene Glycol and to Glycerol

The ingenious method of Jacobs permits, as has been stated, simultaneous measurement of cell permeability to water and to dissolved substances. The necessary data for computation of either are the same.

In preliminary experiments, we attempted to measure the permeability of oyster eggs to ethylene glycol, a solute which because of its convenient rate of

<sup>7</sup> For *Chaetopterus* and *Cumingia* we do not have as yet sufficient data on exosmosis for computation by equation 3. It is of interest, however, to note that permeability values for endosmosis by this equation agree remarkably well with values computed by Jacobs' method for shrinking.

penetration has been a favorite with other types of cells (2, 8). But oyster eggs proved so highly permeable to ethylene glycol that our measurements were not sufficiently accurate. We therefore used a related substance, diethylene glycol, which, as Stewart and Jacobs had shown with *Arbacia* eggs (8), penetrates more slowly. This substance was found suitable for quantitative measurements with oyster eggs; it penetrates into these cells rapidly but at a rate readily measurable by the diffraction method. The values of permeability of this substance are shown in the column next to the last of Table II. The figures express the number of mols  $\times 10^{-15}$  of diethylene glycol which enter the cells per minute through each square micron of surface, at a concentration difference of 1 mol per liter; the average value is 27.2.

In other experiments, glycerol was chosen as the solute to be studied, since we had previously observed that this substance penetrates very slowly into one type of cell, the *Arbacia* egg, whereas it penetrates readily into another, the *Chaetopterus* egg. The oyster egg proved still more permeable to glycerol; in three experiments its permeability averaged 20.7.

#### 6. Comparison of Permeability Values

It may now be of interest to compare the values of permeability obtained for the oyster egg with values previously obtained by us for three other invertebrate eggs. This comparison is restricted to determinations made under similar experimental conditions, using the same technique of measuring cell volume and the same methods for computing permeability. The values are collected in Table III. Inspection of the table brings out first, that for each of the four types of cells permeability to water computed from the course of swelling (by equation 3) is in good agreement with values obtained from the course of shrinking by Jacobs' method; with the exception of the slightly higher permeability obtained by Jacobs' method for *Arbacia*, the determinations for the other cells are almost identical. It follows, as a corollary, that permeability to water is not affected by the presence in the medium (sea water) of the non-electrolytes, ethylene glycol, diethylene glycol, or glycerol; moreover, when the object of experiments is solely evaluation of cell permeability to water, it becomes a matter of convenience which of the two methods is employed.

It will next be noted that the oyster egg is considerably more permeable to water and to solutes than are the other three kinds of marine eggs. Thus, for water the ratio is, very nearly: *Arbacia*, 1; *Chaetopterus*, 4; *Cumingia*, 4; *Ostrea*, 6.

Ethylene glycol penetrates three of the cells in approximately the same ratio as water, but for oyster eggs the ratio is probably much higher. Diethylene glycol has the very high permeability of 27 for *Ostrea*; this is ten times the value obtained by Stewart and Jacobs for *Arbacia* (8).

Glycerol penetrates into the *Arbacia* egg at an extremely slow rate; hence



accurate determinations of time required to attain minimum value are difficult, and the present permeability value must be regarded as an approximation. But *Chaetopterus* is freely permeable to glycerol, more permeable in fact than is *Arbacia* to the readily penetrating ethylene glycol. *Ostrea*, by contrast, is only three times as permeable as *Chaetopterus*. Taking the value for *Arbacia* as sufficiently accurate for obtaining ratios, we find the following: *Arbacia*, 1; *Chaetopterus*, 210, and *Ostrea*, 690.

From these several comparisons it becomes evident that the relative ease of penetration of water is not paralleled by permeability to solutes. For every solute, as well as for water, the permeability of the four kinds of cells is specific. This finding is in harmony with observations made on other kinds of living cells (9).

TABLE III

Comparative permeability to water, and to ethylene glycol, diethylene glycol, and glycerol. Values for water are given in  $\mu^3/\text{min.}/\mu^2/\text{atmosphere}$ ; they are computed from the course of swelling by equation 3 (1st method), and from the course of shrinking by Jacobs method (2nd method). Permeability constants for the solutes are in  $\text{mols} \times 10^{-15}/\text{min.}/\mu^2/\text{mols per liter}$ . All values are based on measurements made by the diffraction method at a temperature of  $22^\circ \pm 0.5^\circ\text{C.}$ ; values for *Arbacia*, *Chaetopterus*, and *Cumingia* have been taken from previous publications (2, 4).

Kind of egg	Water (1st method)	Water (2nd method)	Ethylene glycol	Diethylene glycol	Glycerol
<i>Arbacia</i> .....	0.10; 0.12	0.17	3.5		0.03
<i>Chaetopterus</i> .....	0.44	0.46	14.3		6.3
<i>Cumingia</i> .....	0.46	0.41	15.6		
<i>Ostrea</i> ....	0.60	0.6; 0.7	(Very rapid penetration)	27.2	20.7

## SUMMARY

Investigations of the osmotic properties of oyster eggs by a diffraction method for measuring volumes have led to the following conclusions:

1. The product of cell volume and osmotic pressure is approximately constant, if allowance is made for osmotically inactive cell contents (law of Boyle-van't Hoff). The space occupied by osmotically inactive averages 44 per cent of cell volume.

2. Volume changes over a wide range of pressures are reversible, indicating that the semipermeability of the cell during such changes remains intact.

3. The kinetics of endosmosis and of exosmosis are described by the equation,  $\frac{dV}{dt} = K \cdot S \cdot (P - P_0)$ , where  $dV$  is rate of volume change;  $S$ , surface area of cell,  $(P - P_0)$ , the difference in osmotic pressure between cell interior and medium, and  $K$ , the permeability of the cell to water.

4. Permeability to water during endosmosis is  $0.6\mu^3$  of water per minute, per square micron of cell surface, per atmosphere of pressure. The value of permeability for exosmosis is closely the same; in this respect the egg cell of the oyster appears to be a more perfect osmometer than the other marine cells which have been studied. Permeability to water computed by the equation given above is in good agreement with computations by the entirely different method devised by Jacobs.

5. Permeability to diethylene glycol averages 27.2, and to glycerol 20.7. These values express the number of mols  $\times 10^{-15}$  which enter per minute through each square micron of cell surface at a concentration difference of 1 mol per liter and a temperature of  $22.5^\circ\text{C}$ .

6. Values for permeability to water and to the solutes tested are considerably higher for the oyster egg than for other forms of marine eggs previously examined.

7. The oyster egg because of its high degree of permeability is a natural osmometer particularly suitable for the study of the less readily penetrating solutes.

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# ON THE PRODUCTION AND USE OF PERMANENTLY ALTERED STRAINS OF YEAST FOR STUDIES OF IN VIVO METABOLIC ORGANIZATION

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## I

The nature of the dynamic organization of the various chemical units found within the living cell by use of *in vitro* methods is obviously one of the more important steps in the investigation of the metabolic machinery of the cell. In the attack on this aspect of cellular biochemistry it is obviously desirable that the cell remain alive (intact) during the course of experiments designed to reveal the *in vivo* interrelationships of the components of its various metabolic systems. Unfortunately few experimental procedures are available which are definitely known to fulfill this condition.

A most valuable tool for the *in vivo* attack on mechanisms of cellular metabolism has been, thus far, the so-called "specific" inhibitor. The use of such compounds as KCN, CO, NaF, and iodoacetic acid to block certain enzymic activities within the living cell has provided data which initially guided the isolation and purification of various cellular enzymes and the *in vitro* reconstruction of our present models of cellular metabolic mechanisms. Some of these inhibitors, however, were found to be non-specific in their action, thus limiting their usefulness for the *in vivo* attack on metabolic mechanisms. Cyanide, for example, in a range of moderate concentrations reversibly blocks the cytochrome—cytochrome-oxidase complex and inhibits oxygen consumption. In a range of low concentrations, however, cyanide may stimulate oxygen consumption and bring about a marked increase in the rate of respiration. (See Commoner, 1940, for details and for other examples.)

The following properties of specific inhibitors have also limited their general usefulness:

(a) The inhibitor may form an inactive complex with certain extracellular substrates employed in the experiments (Green and Williamson, 1936).

(b) An inhibitor may simultaneously affect more than one metabolic system within the cell even in experiments made under conditions which are believed to "isolate" single metabolic activities. Cyanide, for instance, has been shown to produce a distinct inhibition of fermentation as well as respiration in yeast held under non-

proliferating conditions in sugar-containing phosphate buffer solutions (Warburg, 1925*b*).

(*c*) The possible reversibility of the inhibitory effect in the course of an experiment may be an added complicating factor. For cyanide it has been shown that the permanence of the inhibitory effect depends on the concentration of the inhibitor and the time it remains in contact with the cells. (See Warburg 1925*a*; Buchanan, 1926.)

(*d*) The slow penetration of the inhibitor into the cell may produce an effect which is a function of time (for cases involving iodoacetate inhibition see Saslow, 1937; for fluoride inhibition, Runnström and Sperber, 1938).

(*e*) It is technically difficult to maintain a constant concentration of such inhibitors as HCN during the course of experiments. With cyanide, compensation must be made for the tendency of HCN to distill out of experimental cell suspensions into the alkaline CO<sub>2</sub> absorber used in measuring the rate of O<sub>2</sub> uptake (Krebs, 1935; Robbie, Boell, and Bodine, 1938).

## II

A method of circumventing some of the difficulties encountered in working with inhibitors has been on trial in this laboratory for several years. The method consists of treating a strain of yeast during cell proliferation with a specific inhibitor until a new stable strain of yeast possessing characteristics different from those of the original (parent) strain, and capable of maintaining the altered characteristics in the absence of the inhibitor, is obtained. Our technique was adapted from the earlier work of Meissel (1933). For other contributions on the effect of inhibitors on proliferating yeast see Pett (1936), and Yoshikawa (1938).

Our first attempt with KCN as the modifying agent has yielded a pure sub-strain which exhibits metabolic properties generally characteristic of yeast cells poisoned with KCN under non-proliferating conditions. The particular characteristics of this strain have remained constant for a period of 5 years during cultivation on media free from cyanide.

Our cyanide substrain does not have cytochrome activity, according to the tests employed, and is apparently entirely lacking in certain components of the enzyme complex believed to be responsible for the major portion of the oxygen consumption of its parent strain. A final pronouncement on this point depends, however, on the development of a satisfactory extraction and assay method for the cytochrome-oxidase component of yeast cells.

The disappearance of cytochrome-oxidase activity upon proliferation of the cells in contact with cyanide indicates a non-reversibility of the action of cyanide. This has not previously been definitely established by experiments under the usual non-proliferating conditions. It thus appears from our results that cyanide can bring about a temporary (reversible) inhibition of oxidase activity under non-proliferating conditions whereas, under proliferating conditions it acts irreversibly destroying the ability of growing cells to synthesize cytochrome-oxidase in an active form or at all.

The exact nature of the action of cyanide in bringing about the permanent (genetic) alteration has not been investigated as yet; neither have we studied the possibility of replacing the altered, or lost, enzyme units by (a) culture procedures (see Lwoff, 1933), (b) the addition of various isolated cell compo-

TABLE I

*Comparison of the Metabolic Characteristics of the Parent Strain with Those of the Cyanide Substrain of Yeast*

	Cyanide substrain	Parent strain
Respiration		
$Q_{O_2}$ in 5 per cent dextrose . . . . .	4.7	31.8
Fermentation		
Aerobic ( $Q_{CO_2}^{air} - Q_{O_2}^{air}$ ) . . . . .	238.5	156.5
Anaerobic ( $Q_{CO_2}^{N_2}$ ) . . . . .	248.8	192.8
Cytochrome-oxidase tests*. . . . .	Negative	Positive
Cytochrome c absorption bands. . . . .	Do not disappear in oxygen	Disappear in oxygen
Catalase test. . . . .	Positive	Positive
Glucose dehydrogenase test†. . . . .	Positive	Positive
Effect of inhibitors on respiration		
KCN. . . . .	No effect	Complete inhibition
NaN <sub>3</sub> . . . . .	No effect	Complete inhibition

\* Procedures employed were those of Keilin (1925): (a) Nadi reagent; (b) manometric method employing *p*-phenylenediamine hydrochloride.

† Anaerobic methylene blue procedure carried out in Thunberg tubes. The dehydrogenase activity was the same in both strains.

*Addendum.*—

#### *Production of the Cyanide-Substrain*

The procedure employed during 1935–36 in obtaining our cyanide substrain was briefly as follows: Cells of a pure strain of *Saccharomyces cerevisiae* Hansen (American Type Culture Collection No. 4360), originally derived from a single cell, were seeded into 1.5 per cent Difco malt extract broth containing an initial KCN concentration of  $m/1000$ . The culture was incubated for 48 hours at 25°C., then washed on the centrifuge with sterile  $m/15$  KH<sub>2</sub>PO<sub>4</sub>, and subcultured in a modified Williams' medium (see Castor, 1939). After a series of liquid subcultures 3 single cell isolations were made and each one maintained on agar slants. Since the respiratory metabolism of the yeast from each isolation was found to be identical we finally selected one of the pure lines and have subsequently carried it on agar slants. Pure culture conditions were rigorously maintained throughout all the procedures.

The results on the cyanide substrain reported in Table I were recently obtained on yeast grown in a liquid subculture.

nents during non-proliferating conditions (Ogston and Green, 1935), or (c) the genetic methods developed for yeast by Winge and Laustsen (1938). These aspects of the problem will be the subject of future investigations.

The absence of cytochrome-oxidase and cytochrome c activity in cells of the cyanide substrain, together with the presence of a small cyanide- and azide-stable respiration (about 15 per cent. of the respiration of the parent strain)

makes this new strain of yeast of considerable value for direct tests of the rôle of flavoprotein in cyanide-insensitive respiration. It should also be useful for investigations of the *in vivo* activities of the cytochrome components a and b. Other examples of its advantages in investigations of intracellular metabolic mechanisms will readily suggest themselves.

### III

The inhibitor-produced substrain of yeast thus makes available an unlimited supply of cells which possess a range of metabolic characteristics, and presumably an *in vivo* chemical organization, markedly different from that of its parent strain of yeast. More important, however, is the possibility of analyzing these cells, either directly by chemical methods, or indirectly by physiological methods, for their component enzyme systems without destroying the altered strain of yeast, and especially, without interference from the chemical agent originally employed in the modification.

It is our plan, as time permits, to attempt the production of other strains of *Saccharomyces cerevisiae* and other yeasts by use of various specific enzyme poisons and various physical agents which modify cellular metabolism. It is hoped that by this method we will be able to delete from, or modify in, the cell its various constitutive enzymes or enzyme systems and ascertain their probable rôle in the chemical organization of cells of the parent strain. It should be noted, of course, that the characteristic metabolic activities of the "normal" parent cells depend upon the usual functional organization of the full complement of the component enzyme systems of the cell. Changes in metabolic characteristics produced by the elimination or alteration of any cellular component may be not solely the expression of the loss of a particular enzyme unit, but rather the expression of a fundamentally new dynamic organization resulting from the deletion. In the latter case the results obtained by this program of research would be at variance with the usual *in vitro* reconstructions. So far, however, the results obtained with our cyanide substrain are in accord in most essentials with the *in vitro* reconstructions of Keilin and Warburg.

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# THE VISUAL SYSTEMS OF EURYHALINE FISHES

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The red photosensitive pigment rhodopsin participates with the carotenoids retinene<sub>1</sub> and vitamin A<sub>1</sub> in a cycle found almost universally in the rods of vertebrate retinas. All terrestrial vertebrates and all marine fishes so far examined except the Labridae possess this system. The true freshwater fishes, however, have evolved a different retinal cycle, compounded of the purple photopigment porphyropsin, with retinene<sub>2</sub> and vitamin A<sub>2</sub> (Wald, 1936-39).

For this reason a special interest attaches to the situation in the euryhaline fishes, which, though usually restricted in spawning either to the sea (catadromous) or to fresh water (anadromous) can exist otherwise in both environments. One may expect a distinctive distribution of visual systems within this group; and these animals alone, since they pass readily from one environment to the other, permit a test of whether the visual system is fixed genetically or varies with the history of the individual.

A survey of the eye and liver vitamins A of several euryhaline fishes has already been reported (Wald, 1938-39*a*). The retinas contain either mixtures of both vitamins A, in which that commonly associated with the spawning environment predominates; or exclusively the spawning type of vitamin A. The vitamin A configurations of both eye tissues and liver are primarily determined genetically, and are relatively independent of the environment.

The present paper is concerned with an examination of the visual systems of certain euryhaline fishes: the anadromous salmonids, white perch and alewife; the catadromous eel; and the killifish, which verges on the catadromous condition.

## I. Methods

The methods used in this investigation are identical with those described previously (Wald, 1936-39). Photopigments were extracted with aqueous digitonin solution from dark adapted retinas previously hardened with alum and neutralized with phosphate buffer. The absorption spectrum of rhodopsin prepared in this way is maximal at about 500 m $\mu$ , that of porphyropsin at about 522 m $\mu$ .

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The carotenoid components of the retinal cycle were examined in a standardized procedure, which yielded the data shown in Figs. 1, 2, 4, and 6. Retinas were extracted by shaking with petroleum ether (benzine) in three singular states: (1) dark adapted; (2) immediately on exposure to bright light, which bleaches the photopigments to orange products; and (3) after 1 hour at room temperature following irradiation, during which the retinas fade to colorlessness. In stenohaline marine or fresh water fishes respectively, the dark adapted tissues yield small residual amounts of either vitamin  $A_1$  or  $A_2$ . The just irradiated retinas yield large quantities of retinene<sub>1</sub> or retinene<sub>2</sub>, liberated in the bleaching of photopigment. Extracts of bleached and completely faded retinas contain large amounts of vitamins  $A_1$  or  $A_2$ , formed from the retinenes during the fading process. In each case, the benzene extract was transferred to chloroform and tested by mixing with antimony chloride reagent. In this reaction, the retinenes and vitamins A yield blue colorations due to absorption bands in specific positions: retinene<sub>1</sub> at 664 m $\mu$ , retinene<sub>2</sub> at 705 m $\mu$ , vitamin  $A_1$  at 615–620 m $\mu$ , and vitamin  $A_2$  at 693–696 m $\mu$ .

All spectra but those of Fig. 7 were recorded automatically with Hardy's photoelectric spectrophotometer at the Massachusetts Institute of Technology. The spectra of Fig. 7 are transcriptions of similar recordings. Absorptions are expressed as percentage absorption,  $1 - I/I_0$ , in which  $I_0$  is the incident and  $I$  the transmitted intensity; or as extinction or optical density,  $\log I_0/I$ .

## II. Salmonids

Members of three different genera of the family Salmonidae have been examined: the brook trout, *Salvelinus fontinalis*; the rainbow trout, *Salmo irideus*; and the chinook or king salmon, *Oncorhynchus tshawytscha*. All these fish are anadromous and hence potentially migratory. All of them, however, can, and on occasion do pass their entire life cycles without detriment in fresh water. They are to be regarded as fresh water fishes which—since euryhaline—can migrate seaward. The chinook salmon performs this migration commonly, the brook trout more rarely, becoming in the process a sea trout, and the rainbow trout most rarely of all. It should be noted, however, that the fish used in the following experiments were obtained from a hatchery, and had never left fresh water.

These fish all possess mixtures of the rhodopsin and porphyropsin systems, predominantly the latter. This is shown most clearly in tests of the retinal carotenoids. Data from the brook trout and salmon are shown in Figs. 1 and 2; those from the rainbow trout are very similar in appearance. Extracts of dark adapted retinas, tested with antimony chloride, yield low absorption spectra, displaying a small vitamin  $A_1$  inflection at about 620 m $\mu$  and a much higher vitamin  $A_2$  band at about 696 m $\mu$  (Figs. 1 and 2, curves *a*). Similar extracts of just irradiated retinas in which the photopigments had been bleached to orange products yield high, peculiarly broad bands, maximal at 686–691 m $\mu$ , due to fusion of the absorption bands from retinene<sub>1</sub> and retinene<sub>2</sub>, at 664 and 705 m $\mu$  respectively (curves *b*). Bleached retinas which had been allowed to

fade before extraction yield high double-banded absorptions, displaying a large vitamin A<sub>2</sub> and a smaller vitamin A<sub>1</sub> component (curves *c*). The location of the vitamin A<sub>1</sub> band on the shoulder of the vitamin A<sub>2</sub> absorption shifts its maximum somewhat toward the red. The proportions of both vitamins in these extracts have already been reported (Wald, 1938-39*a*). Clearly both

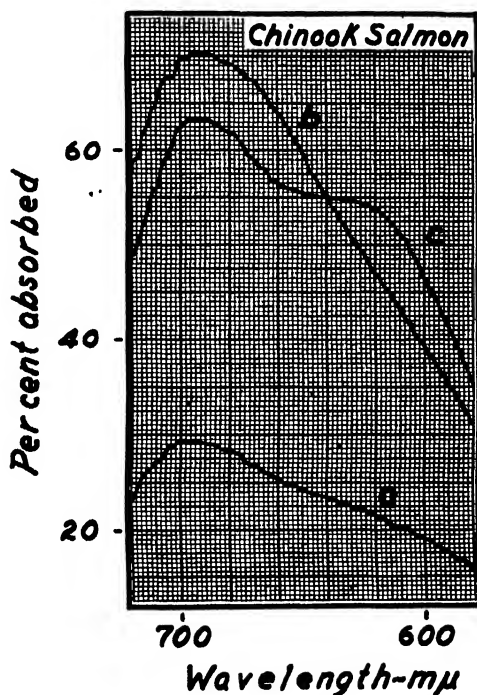


FIG. 1. Spectra of the antimony chloride reaction with benzene extracts of seven chinook salmon retinas: (*a*) dark adapted; (*b*) on irradiation with bright daylight; and (*c*) after 1 hour at room temperature following irradiation.

of them, but predominantly vitamin A<sub>2</sub>, participate actively in the retinal cycles of these fishes.

The duplex character of these visual systems is reflected also in the absorption spectra of their photopigments (Fig. 3). The photopigment absorption maxima occur at 510-515 mμ, approximately halfway between those of rhodopsin (500 mμ) and of porphyropsin (522 mμ). They represent mixtures of both pigments.

The bleaching of brook trout photopigment is also shown in Fig. 3. This is intermediate at all stages between the bleaching of rhodopsin and of porphyropsin described previously (Wald, 1937-38; 1938-39*b*).

### III. Eel and Killifish

The so called freshwater eel, *Anguilla rostrata*, is a typically catadromous fish. It spawns in sea water of great depth and salinity, and ordinarily enters fresh water after more than a year of marine existence. Its history therefore is directly the reverse of that of the salmonids—it is essentially a marine fish which euryhalinity permits to migrate into fresh water.

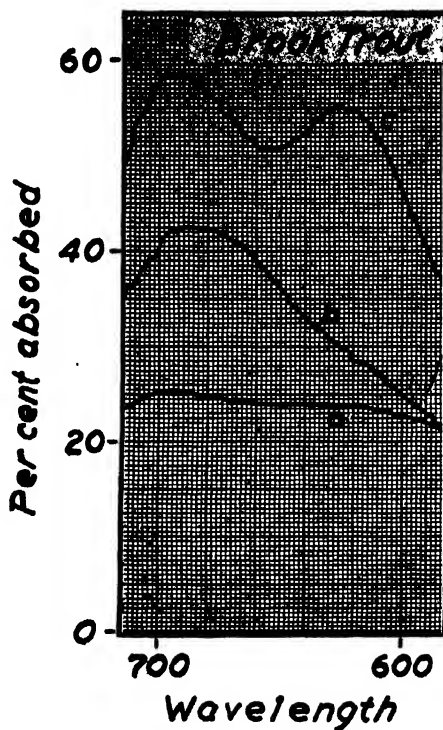


FIG. 2. Spectra of the antimony chloride reaction with extracts of seven brook trout retinas. Otherwise as in Fig. 1. Curve *a* is raised above its correct level due to turbidity of the solution.

The eel, like the salmonids, possesses a mixture of the rhodopsin and porphyropsin systems, but in reverse proportions. This is demonstrated in Fig. 4. Each of the spectra in this figure resembles a mirror-image of its analogue in Figs. 1 and 2. Curves *a* and *c* display the bands of both vitamins A, in this case with the A<sub>1</sub> band dominant and in the correct position, while the A<sub>2</sub> band is shifted toward shorter wavelengths by its location on the rising limb of the A<sub>1</sub> absorption. Similarly, the maximum of the fused band of the retinenes (curves *c*) occurs in this case at 672–674 mμ, close to the band of the dominant retinene<sub>1</sub>.

Absorption spectra of eel photopigment solutions are shown in Figs. 5 and 7. Though these preparations contain an admixture of porphyropsin, their maxima occur at 498 and 502  $m\mu$ , within the range of rhodopsin maxima. The bleach-

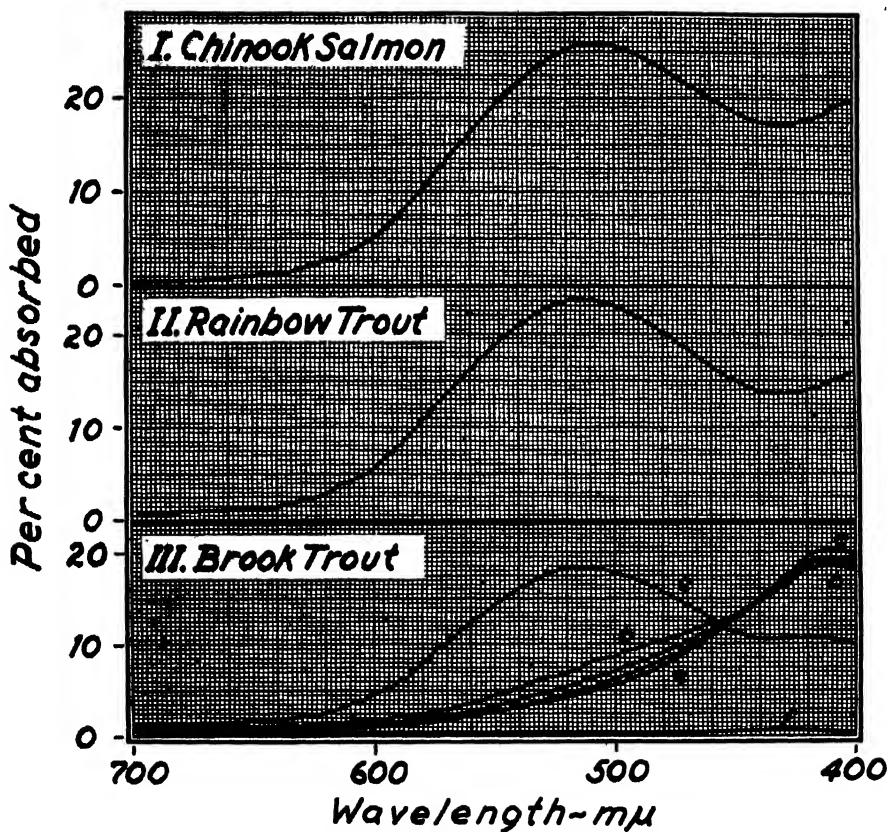


FIG. 3. Spectra of photopigments from salmonid retinas. All these fish were yearlings, about 7-8 inches long. The total extracts in 4 cc. of digitonin solution were measured in a layer 1 cm. thick. I. Eight chinook salmon retinas. II. Six rainbow trout retinas. III. *a*, six brook trout retinas. This solution was exposed to bright light for  $\frac{1}{2}$  minute and its spectrum thereafter re-measured in darkness, *b* at 1.7 minutes, *c* at 4.5, *d* at 13.7, and *e* at 60.5 minutes (26.7°C.). *f* is the control spectrum of the solvent alone.

ing of eel photopigment, shown in Fig. 5, also closely resembles that of rhodopsin.

It is clear that the eel, in accord with its catadromous nature, possesses a mixture of both the rhodopsin and porphyropsin systems, with a great predominance of the former.

The killifish, *Fundulus heteroclitus*, is remarkable in being euryhaline both as embryo and adult. Indeed its embryo is reported to develop and hatch normally even in distilled water (Loeb and Cattell, 1915). Conceivably, therefore, this fish might spawn in either salt or fresh water, and so cannot be classed strictly as either anadromous or catadromous.<sup>1</sup> Actually it inclines markedly throughout its life cycle toward the sea. It is commonly regarded

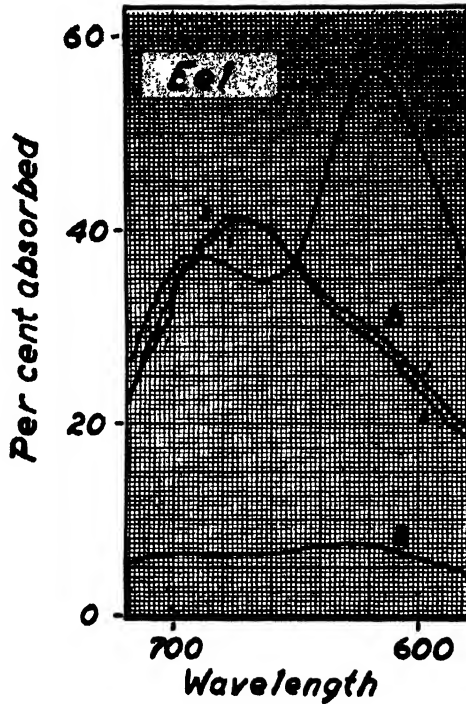


FIG. 4 Spectra of the antimony chloride reaction with extracts of seven eel retinas. Otherwise as in Fig. 1. Due to fluctuation in the spectrophotometer record, curve *b* was re-drawn.

as marine, and only rarely penetrates to fresh water. I know of no record of its spawning in fresh water. Ordinarily it maintains a stable if equivocal position close to shore and in tidal parts of streams; and this appears to be due as much to a predilection for shallow as for brackish water (*cf.* Bigelow and Welsh, 1924). In fresh water in the laboratory, it usually survives only a matter of several weeks, but this circumstance is complicated by its great sus-

<sup>1</sup> Dr. E. N. Warner of Ohio State University informs me, however, that he has attempted to fertilize *Fundulus* eggs in tap water without success, apparently because this medium rapidly inactivates the sperm.

ceptibility while in this medium to fungal attack, notably by *Saprolegnia* (Sumner, 1905). Among euryhaline fishes it is certainly much closer to the catadromous than to the anadromous way of life.

The visual system of *Fundulus* closely resembles that of the eel. A spectrum of its photopigment was published among those of permanently marine fishes before its peculiar status had been appreciated; significantly, in this collection

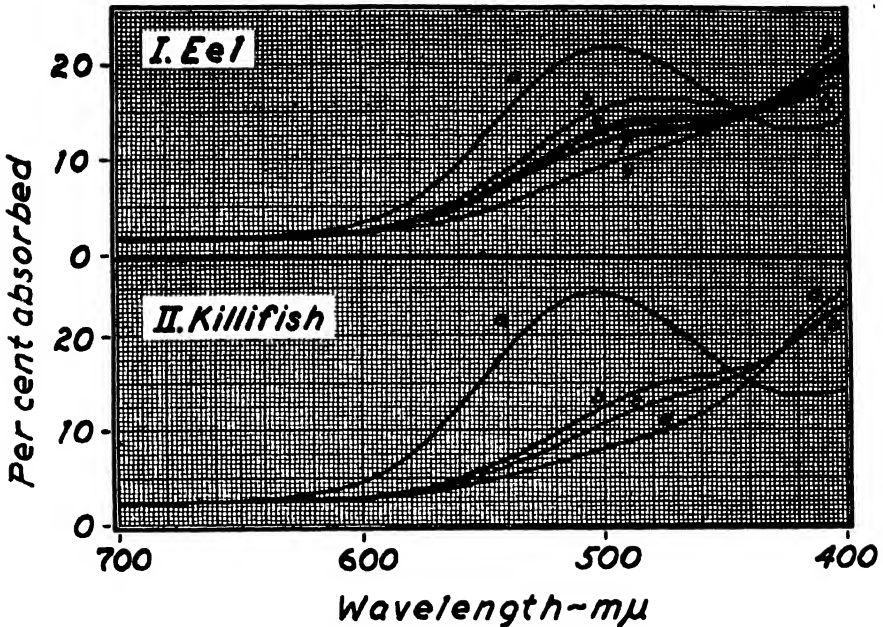


FIG. 5. I. *a*, Spectrum of photopigment from the retinas of a single eel, 26 inches long, in 4 cc. of digitonin solution, measured in a 1 cm. layer. Following 20 seconds exposure to bright light, the spectrum was re-measured in darkness, *b* at 1.5 minutes, *c* at 4.0, *d* at 6.3, *e* at 17.7, and *f* at 37 minutes. The preparation was re-irradiated or 20 seconds, and at 39.5 minutes yielded curve *g* (pH 6.3; 37°C.) II. *a*, Spectrum of photopigment from ten killifish. This preparation was irradiated for 30 seconds, and spectra re-drawn in darkness, *b* at 1.7, *c* at 4.0, and *d* at 31 minutes (pH 7.0).

of otherwise pure rhodopsin spectra that of *Fundulus* occupies the extreme long wavelength position, with a maximum at 502 mμ (Wald, 1937-38, Fig. 1). The spectrum of another preparation, maximal at about 505 mμ, is shown in Fig. 5. *Fundulus* spectra in general vary within about the same range as those of the eel (Fig. 7). That they represent mixtures of both rhodopsin and porphyropsin is shown by examination of the retinal vitamins A. An extract of bleached and faded retinas tested with antimony chloride displays the bands of both vitamins A<sub>1</sub> and A<sub>2</sub> with the former greatly predominant.



IV. *White Perch and Alewife*

The white perch (*Morone americana*) and the alewife (*Pomolobus pseudo-harengus*) are typically anadromous. Unlike the salmonids each of them possesses close relatives among permanently marine forms. The visual systems of two of the latter have already been described: the black sea bass, a serranid like the white perch; and the common herring, a clupeid like the alewife (Wald, 1936-37; 1938-39*a*). Both possess pure rhodopsin systems. It is all the more remarkable that their anadromous kin display precisely the opposite situation.

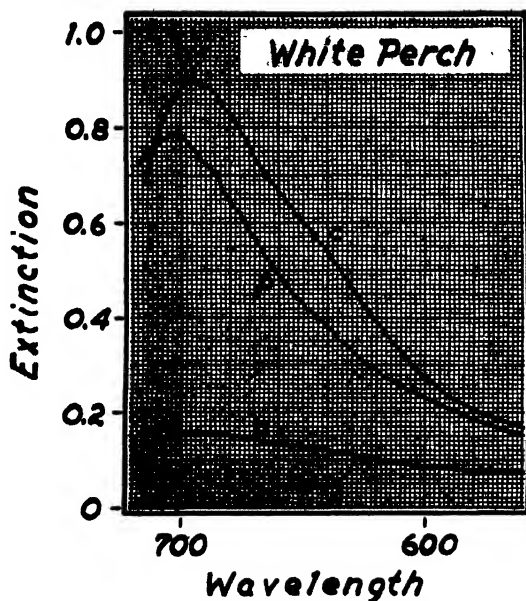


FIG. 6. Spectra of the antimony chloride reaction with extracts of six white perch retinas. Otherwise as in Fig. 1.

The photopigment of the white perch retina has already been examined (Wald, 1938-39*b*). It possesses the usual properties of porphyropsin (Fig. 7). That it includes no admixture of rhodopsin is confirmed by examination of the carotenoid cycle (Fig. 6). This reveals bands due only to vitamin  $A_2$  and retinene<sub>2</sub>, with no trace of their analogues in the rhodopsin system.

In the alewife only extracts of light adapted retinas to which the pigment epithelia were attached have been examined. They show the presence of vitamin  $A_2$  alone, with no trace of  $A_1$  (Wald, 1938-39*a*). This observation has been confirmed repeatedly with fish taken from brackish water at the beginning of their spawning migration from the sea, and therefore in a condition which might maximally favor the presence of vitamin  $A_1$ .

## V. DISCUSSION

It may be concluded that many euryhaline fishes give retinal evidence of their euryhalinity in possessing mixtures of the rhodopsin and porphyropsin systems; and of their anadromous or catadromous natures in having predominantly the system ordinarily associated with the spawning environment. The latter tendency is pursued to completion in fishes like the white perch and alewife, which possess the spawning-type of visual system alone. A remark-

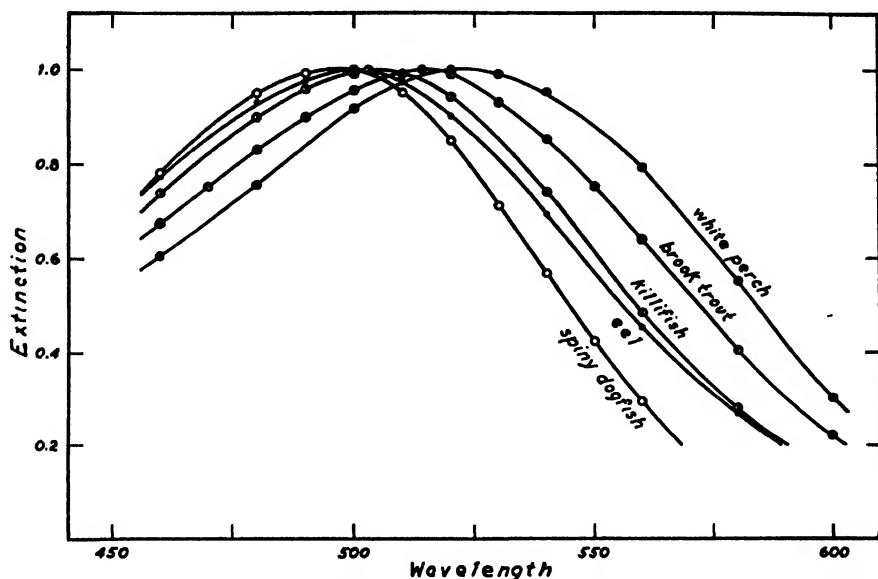


FIG. 7. Spectra of photopigments from various fishes, illustrating the transition from an exclusively rhodopsin to an exclusively porphyropsin system. The permanently marine dogfish possesses rhodopsin alone, the catadromous eel and approximately catadromous killifish predominantly rhodopsin, the anadromous brook trout predominantly porphyropsin, and the anadromous white perch porphyropsin alone.

able parallelism obtains, therefore, between a graded series of salinity relations and of retinal components. This is illustrated in Fig. 7. Beginning with rhodopsin from a permanently marine elasmobranch, the spectrum of retinal photopigment precesses regularly toward longer wavelengths in preparations from the eel, a marine fish which migrates into fresh water; the killifish, a stable brackish water form; the brook trout, a fresh water fish which enters the sea; and the white perch, an anadromous fish which has completed the transition to porphyropsin.

It is important to recall that the Labridae, all permanently marine, constitute one already known exception in this distribution. The retinas of the tautog

(*Tautoga onitis*) and cunner (*Tautoglabrus adspersus*), like those of anadromous forms, contain a great predominance of vitamin A<sub>2</sub> (Wald, 1938-39 *a*; unpublished observations). Yet both are obligate marine fish. I have confirmed Sumner's observation (1905) that both species die within several hours in fresh water. The linkage between osmotic habit and type of vitamin A metabolism, therefore, while remarkably complete, is not absolute.

These patterns are genetic, and to a first approximation independent of the environment (Wald, 1938-39 *a*). The duplexity of visual systems of salmonids raised exclusively in fresh water; the great predominance of rhodopsin in the eel after years of fresh water existence, and of porphyropsin in the permanently marine Labridae; the directly opposed systems of the closely related herring and alewife after long periods in the sea; and other similar observations all lead to this conclusion. Whatever genetic factors govern the salinity relations of these animals simultaneously determine the composition of their visual systems.

The latter have therefore a phylogenetic rather than an ontogenetic significance. In this sense, it is possible that the euryhaline fishes represent transitional stages in evolutionary migrations between fresh water and the sea. This view, if valid at all, furnishes no hint of the direction of migration. Disregarding other types of information, the eel for example might equally well be regarded as a marine fish which has become euryhaline preparatory to its transition to fresh water; or as a fish which, having all but completed migration to the sea, retains euryhalinity as a vestige of previous fresh water existence.

Finally the euryhaline fishes provide a retinal situation of peculiar theoretical interest for color vision. Rhodopsin and porphyropsin, both rod pigments, probably play no direct role in this function; yet they provide an exact model of the kind of variation in visual systems which should meet the peripheral requirements of hue discrimination (Wald, 1938-39 *b*). The model is completed in those euryhaline fishes which possess mixtures of both systems. In this respect, though possibly in no other, these animals have satisfied one basic requirement of dichromatic vision.

#### SUMMARY

1. The retinas of all marine fishes so far examined except the Labridae, and of all terrestrial vertebrates contain the rhodopsin system alone; those of fresh water fishes the porphyropsin system alone. In the present paper the visual systems of a number of euryhaline fishes are examined—fishes capable of existence in a wide range of salinities, though usually restricted in spawning either to the sea (catadromous) or to fresh water (anadromous).

2. The retinas of the anadromous salmonids (brook trout, rainbow trout, and chinook salmon) contain mixtures of the rhodopsin and porphyropsin systems, predominantly the latter. The retinas of the catadromous eel and the killifish also contain mixtures of both systems, but in reverse proportions.

The retinas of the anadromous white perch and alewife contain the porphyropsin system alone.

3. There is therefore an extensive parallelism between the salinity relations of these animals and the composition of their visual systems. All of them possess predominantly or exclusively the visual system commonly associated with the environment in which the fish spawns.

4. These patterns are fixed genetically, and are to a first approximation independent of the history of the individual. They may represent transitional stages in the evolutionary migration of fishes to and from the sea. The presence of both types of visual system in the retinas of some euryhaline fishes incidentally satisfies one formal requirement of two-component color vision.

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# THE EFFECT OF TEMPERATURE AND OF LYSIN CONCENTRATION ON THE ACCELERATION OF HEMOLYSIS\*

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This paper, which contains a continuation of work described in two others (Ponder, 1939; Ponder and Hyman, 1939), falls into two parts. The first deals with the effect of temperature on the magnitude of the acceleration of hemolysis; the second deals with the behavior of the "acceleration constant"  $R$  when the asymptotes of the time-dilution curves for the standard system and for the accelerated system are approached, and with some curious relations which arise.

## 1. The Effect of Temperature

To find the effect of temperature on the acceleration of hemolysis produced by benzene derivatives, I have set up systems containing 0.8 cc. of various dilutions of saponin (1 in 10,000, 1 in 20,000, etc.), 0.8 cc. of saline (1 per cent NaCl), and 0.4 cc. of a standard suspension (the thrice washed cells of defibrinated rabbit blood finally suspended in 20 cc. of saline). The times for hemolysis in these systems give the standard curve (S in Fig. 1). A second set of systems is set up, the only difference being that 0.8 cc. of an accelerator (e.g. 10 mm/l. benzene) is substituted for the 0.8 cc. of saline in the standard systems. The times for complete hemolysis in this second set of systems give the accelerated curve (A in Fig. 1).

Standard curves and accelerated curves can be obtained at different temperatures by using a series of water baths (12°C., 25°C., 39°C., etc.); in this way all the observations can be made on one sample of blood. The curves are subsequently analyzed, and Fig. 1 shows in a general way how  $R$ , a constant which measures the acceleration, is obtained (see Ponder, 1934 a).

The results for three quite powerful accelerators are shown in Table I. It should be pointed out that the values of  $R$  which are given are those in the range in which  $R$  is constant, and in which the concentration of lysin is relatively great (see below). Each  $R$  value represents the mean of from 5 to 7 points on the curve. All the accelerators were in solution in 1 per cent NaCl.

\* This investigation was carried out under a grant from the Simon Baruch Foundation.

In each case the value of  $R$  shows a negative temperature coefficient. Since the evidence is that the acceleration of lysis takes place in the neighborhood of the cell surfaces, and since the amount of acceleration is generally proportional to the amount of accelerator, the result points to the accelerators being concentrated at the cell surface by a process which has a negative temperature coefficient (followed by a combination with membrane components and with lysin).

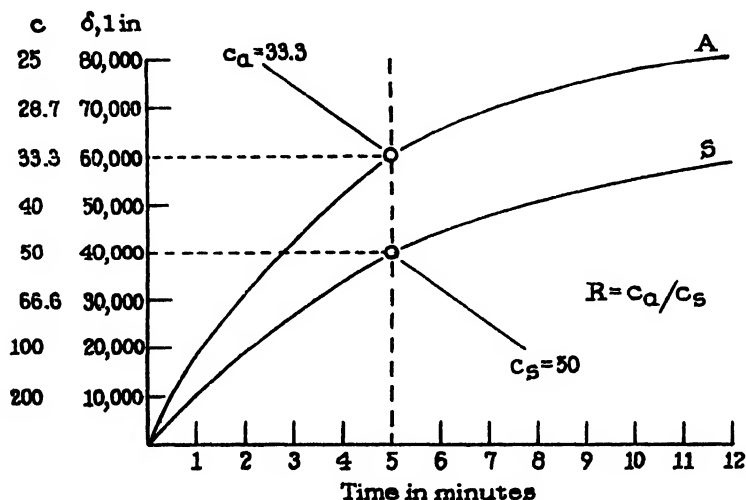


FIG. 1. Ordinate, lysin dilution and concentration in micrograms; abscissa, time in minutes. Standard time-dilution curve marked S, and accelerated curve marked A. The dotted lines show two concentrations which produce lysis in the same time, from the ratio of which  $R$  is derived.

TABLE I

Substance	Concentration	39°C.	25°C.	12°C.
	<i>mm/l.</i>			
Benzene.....	10	0.74	0.67	0.46
Indol.....	1	0.55	0.32	0.21
Nonyl alcohol.....	0.5	0.73	0.62	0.46

This agrees with conclusions 4 and 5 in a recent paper (Ponder, 1939); *viz.*, that benzene is concentrated at the red cell surface, that it can be washed off without leaving permanent effects if the washing is done immediately, but that irreversible effects remain if some time is allowed to elapse before the washing. Indol is concentrated similarly.

## 2. The Effect of Lysin Concentration

If one uses a lysin such as saponin together with a powerful accelerator such as saturated indol, one can plot a standard time-dilution curve for the saponin

alone and an accelerated curve for saponin plus indol, and so one can measure the acceleration by evaluating  $R$  in the usual way. It will then be found that  $R$  is substantially constant while the concentration of lysin is greater than the concentration corresponding to the asymptote of the standard curve, but that when the concentration in the system is less than the asymptotic concentration for the standard curve; *i.e.*, when there is not enough lysin to produce lysis *per se* in the absence of the accelerator, the value of  $R$  falls off towards unity. The limit of the analysis is obviously the asymptote of the accelerated time-dilution curve.

This is shown in Table II and in Fig. 2. This figure shows the sharp change in direction of the curve in the neighborhood of a lysin concentration corresponding to the asymptote of the standard curve ( $22\gamma$ ). What impresses one is that from a lysin concentration present in the system originally, and upon which indol acts as an accelerator, of  $2000\gamma$  to about  $33\gamma$ , the acceleration, as measured by  $R$ , is constant and the values fall on a vertical straight line, while all but one of the points below  $33\gamma$  fall on another straight line passing through the origin  $R = 1.0$ . On general principles, I do not think that the results are really represented by two such straight lines, for this involves the idea of *complete* discontinuity, but in the first instance it is convenient to treat them as if they were.

Call the concentration of lysin in the standard system  $c_s$ . If no accelerator is added, plotting values of  $1/c_s$  against the times which they take to produce complete hemolysis gives the standard time-dilution curve. If an accelerator is added, the concentration of lysin, as judged by the time for complete hemolysis, appears to be not  $c_s$ , but the smaller concentration  $c_a$ , which is accelerated or "potentiated" by the accelerator. Let

$$c_a/c_s = R \quad (1)$$

While  $c_a > c_{s\infty}$  (the asymptotic concentration of the standard curve)  $R$  is a constant  $R_0$ , but when  $c_a < c_{s\infty}$ ,  $R$  increases as  $c_a$  decreases; *i.e.*, the acceleration becomes less. The values of  $c_a$  now lie on the straight line passing through  $R = 1.0$ . If the slope of the line is  $k = (c_{s\infty}/1 - R_0)$ ,

$$c_a = k(1 - R) \quad (2a)$$

and

$$R = (1 - c_a/k) \quad (2b)$$

or

$$R = 1 - c_a(1 - R_0)/c_{s\infty} \quad (2c)$$

and so

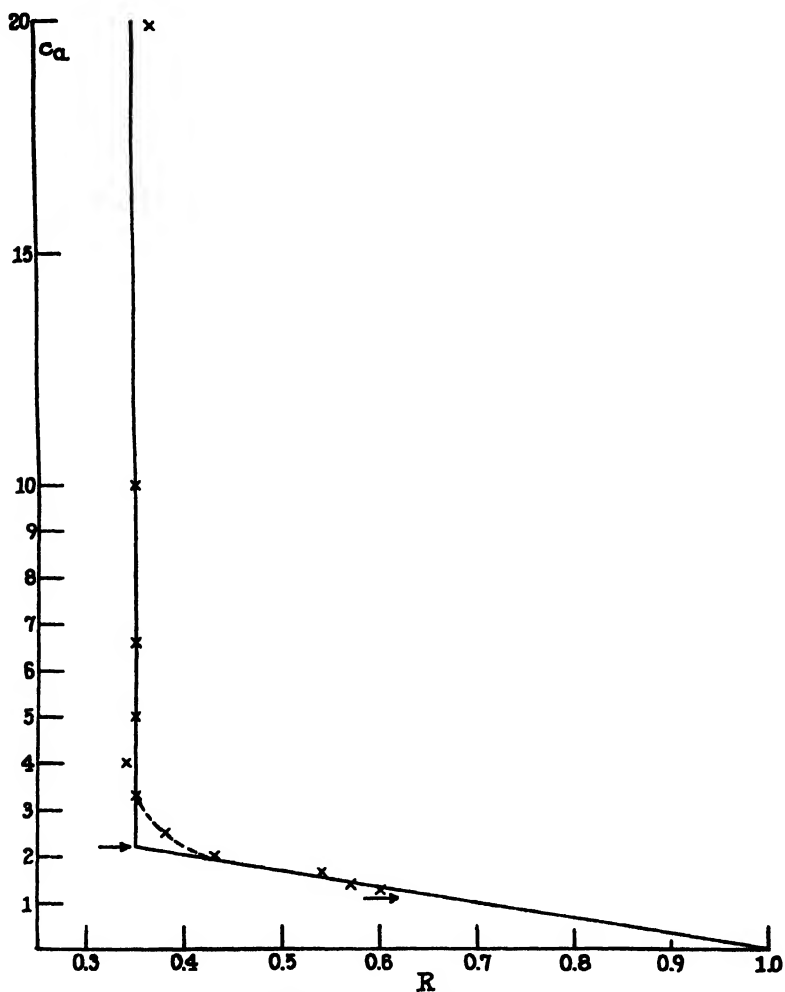
$$c_a = c_s(1 - c_a/k) \quad (3)$$

There must be one value of  $c_a$  which makes  $c_a = c_{s\infty}$  in expression (3); *i.e.*, there must be one concentration of lysin which, when accelerated by the added indol,



TABLE II

Dilution 1 in	$t$ , min. standard	$t$ , min. + indol	$R$
10,000	0.167	0.058	0.37
20,000	0.50	0.109	0.35
30,000	1.4	0.183	0.35
40,000	2.5	0.268	0.35
50,000	5.5	0.350	0.34
60,000	13.0	0.535	0.35
80,000	40.0	1.0	0.38
100,000	> 120.0	3.0	0.43
120,000	—	17.0	0.54
140,000	—	40.0	0.57
160,000	—	100.0	0.60

FIG. 2. Ordinate,  $c_a$ ; abscissa,  $R$ . The crosses represent the values given in

appears to be equal to the asymptotic concentration  $c_{\infty}$  for the standard curve.<sup>1</sup> This value is

$$c_a = 1/(1/c_{\infty} + 1/k) \quad (4)$$

The same conditions must apply to the asymptotic concentrations as to other concentrations less than  $c_{\infty}$ , so

$$c_{\infty} = c_{\infty}(1 - c_a/k) \quad (5)$$

Again there must be one value of  $c_a$  which satisfies the conditions, and this value is the same as that in expression (4). Substitution in expression (5) of this value gives  $c_{\infty}$ , so the concentration of lysin which, when accelerated, appears to be equal to the asymptotic concentration in the standard system is the asymptotic concentration in the accelerated system.

TABLE III

$$c_{\infty} = 22\gamma, \quad R_0 = 0.35$$

$$k = (22/0.65) = 33.9$$

	$c_a, \gamma$	$R$	$(1 - R)$	$c_a/R$
$> c_{\infty}$	22.0	0.350	0.650	63
$1.0 c_{\infty}$	22.0	0.350	0.650	62.9
$0.9 c_{\infty}$	19.8	0.415	0.585	47.7
$0.8 c_{\infty}$	17.6	0.480	0.520	36.7
$0.7 c_{\infty}$	15.4	0.545	0.455	28.5
$0.6 c_{\infty}$	13.2	0.610	0.390	21.6
$0.5 c_{\infty}$	11.0	0.675	0.325	16.3
and so on.				

To set forth these relations in a somewhat different way, let us tabulate in Table III the data contained in Table II.

In an accelerated system the concentration  $c_a$  put into the system together with the accelerator experiences an acceleration  $R$ , and acts as if it were the greater concentration  $c_s = c_a/R$  (expression 1). Looking at the last column of Table III, we see that the concentration apparently acting may be indefinitely greater than  $22\gamma$  when  $c_a$  is great, but that, as  $c_a$  becomes smaller, there comes a point where  $c_a/R = 22\gamma$ , the value of the asymptote  $c_{\infty}$  of the standard system. Expression (4) gives this value of  $c_a$ , which is  $13.33\gamma$ . This corresponds to the asymptote for the accelerated system; if  $c_a$  is less than  $13.33\gamma$ , the value of  $R$

<sup>1</sup> The nomenclature in the literature is neither completely satisfactory nor used uniformly. Sometimes  $c_1$  is used as referring to the standard curve and sometimes to the curve which is compared with it, and the measure of acceleration or inhibition is sometimes given as  $R$ , sometimes as  $1/R$ , and sometimes as  $1 - R$ , as may be convenient. The nomenclature which I have used in this paper is intended to be self-consistent, but even so some of the expressions, such as (4) and (5), are very troublesome to derive.

associated with it is not sufficient to raise its potency to that of 22 $\gamma$ , and complete lysis never occurs.

#### DISCUSSION

The point which obviously requires explanation is that the acceleration  $R$  is constant as long as enough lysin is introduced into the system to produce lysis *per se*, but that if a lesser amount is present the acceleration is reduced in proportion.

The simplest way of approaching the explanation which I propose is to begin with Gorter's observation (1937) that the amount of saponin which is just enough to produce complete hemolysis is just enough to cover the cell surfaces with a monolayer. In the absence of an accelerator, this complete covering will occur when the concentration of lysin is  $c_{\infty}$  or greater; when the concentration is less, covering will be incomplete. Now if we assume that an accelerator produces its effect only at regions of the cell surface at which lysin is acting,<sup>2</sup> the acceleration will be constant for  $c_{\infty}$  and for concentrations greater, but for concentrations less than  $c_{\infty}$ , where there is incomplete covering, the acceleration will be proportionately reduced. This is what is found.

There is, however, considerable doubt as to the interpretation to be placed on Gorter's observation. In 1930 I arrived at a somewhat similar result for the case of sodium oleate; taking the minimum quantity of lysin needed for complete lysis on the one hand, and the dimensions of the oleate molecule on the other, I found that the molecules available for each cell would just about cover the cell surface (Ponder, 1930). But, as in the case of Gorter's result, the figure is a maximum value, for a large proportion of the lysin combines with liberated hemoglobin and other cell contents, and the lysin continues to react with the ghosts of the cells which hemolyze first. Thus if the total amount of lysin needed for complete hemolysis turns out to be just enough to cover the cell surfaces, the reaction which results in lysis must take place at a smaller surface than the whole surface. Further, lysins such as saponin are not taken up by the cells immediately, and a considerable proportion is always to be found in the suspension fluid (Ponder, 1934 *b*, 1935), while the photodynamic lysins erythrosine and eosin, the saponins smilacin and helleborein, and many of the soaps are lytic only in concentrations much greater than required to form monolayers at the surfaces of the cells of the systems. These constitute additional difficulties in the interpretation of Gorter's observation.

Alternatively, let us look at the evidence which suggests that lysis can occur when the cell surface is incompletely covered, the membrane breaking down in

<sup>2</sup> The localization of the action of the accelerator at the cell surface is based on quite good experimental evidence (Ponder, 1926*b*, 1939, and the first section of this paper). In the case of certain inhibitors the situation is different, for the inhibitor reacts with the lysin in the suspension medium (Ponder and Gordon, 1934).

spots, and the hemoglobin escaping through holes. The idea was originally put forward in 1930 by Abramson, who showed that sheep cells sensitized by amboceptor retain their electrical mobility unchanged although they are hemolyzed by the subsequent addition of complement; this led him to suggest that the lytic process may be restricted to certain spots on the surface ("key spots"), too few in number to be detected by a change in mobility. "This hypothesis postulates that lysis takes place at isolated points on the surface rather than by a uniform change in membrane permeability. The hypothesis does not necessarily infer that these spots are structural units arranged in some pattern and picked out by the lysin because of their vulnerability" (Abramson, Gorin, and Ponder, 1940).<sup>3</sup> Much the same point of view has been expressed by Abramson, Furchgott, and Ponder (1938) in a paper describing experiments which show that the mobility of rabbit red cells is not altered when they are hemolyzed by water, freezing and thawing, chloroform, or saponin.<sup>4</sup>

Before this, however, the same kind of conclusion was arrived at in a wholly different way. In 1934 I published preliminary results of a study of the rate of escape of hemoglobin from hemolyzed red cells, and shortly afterwards Fricke (1934) gave a theoretical treatment in which he derived the permeability of the membrane to hemoglobin,  $\mu_H$ , as a function of the time required for a cell to lose 90 per cent of its pigment, or the "fading time." On the assumption that the pigment escapes through  $N$  holes of diameter  $d$  instead of uniformly across the entire membrane, he also obtained the product  $Nd$  as a function of the fading time. Ponder and Marsland (1935) then showed that the fading time is a function of the lysin concentration in the case of saponin, and later Davson and Ponder (1938) found that even in the case of hypotonic hemolysis the fading time depends on the degree of hypotonicity. Ponder and Marsland calculated that when the concentration of lysin is great enough the reaction between the lysin and the membrane component would be so rapid that as much as 10 per cent of the entire membrane might be disintegrated before the end of the fading time, short as it is, but when the lysin concentration is small the situation is different. Ponder and Neurath (1937) placed cells in contact with monolayers of Ca-stearate, and found fading times of about 10-15 seconds, which are about the same as the fading times found for the most dilute saponin systems in which complete lysis occurs. (Again Gorter's idea of the necessity of a monolayer for lysis appears.) But from the fading time the permeability

<sup>3</sup> The complement fixation process is probably of a special type, in which the taking up of the complement is predetermined by the special positions of the antigen-antibody complexes on the surface.

<sup>4</sup> They also found that the mobility of oil droplets or quartz particles completely covered with saponin (0.1 per cent) is more than twice that of ghosts in the same saponin concentration. This point should be borne in mind in discussing the possibility of monolayer formation at the red cell surface.

of the entire membrane,  $\mu_H$ , or  $Nd$ , the number of holes of diameter  $d$ , can be calculated, and for a fading time of 12 seconds  $\mu_H = 0.186(10^{-4})$  cm./sec., or  $Nd = 0.25 \mu$ . This last figure corresponds to one hole of diameter 2500 Å, 10 holes of diameter 250 Å, 100 holes of diameter 25 Å, and so on. Ponder and Neurath suggested 10–20 holes of diameter 250–125 Å as being the most likely to meet the requirements. It should be noticed that the combined area of such holes would be less than 1/10,000th of the cell surface, and so their existence would not be detected by electrophoretic methods. Unfortunately, we could also have areas of permeability of diameter  $d$  and of finite permeability  $\mu_H$  instead of completely permeable holes, for the two possibilities, that of holes and that of increased permeability of the entire membrane, are not mutually exclusive.

There is a close correspondence between these results and those obtained from measurements of conductance (Fricke and Curtis, 1935), for when lysins are used in low concentrations the cells, although hemolyzed, behave as virtual non-conductors with very nearly the same capacity as intact cells. This would be expected if lysis were the result of the formation of holes the combined area of which was of the order of 1/10,000th of the cell surface. Saponin in large concentrations, however, breaks down the membrane so completely that both resistance and capacity disappear.

In the last resort, we have to turn to those cases in which the lysin brings about hemolysis in quantities too small to cover the cell surface. I have already remarked that, despite the results of spreading experiments, there is considerable doubt as to whether the smallest lytic quantities of sodium oleate and of saponin are sufficient to cover the cell surface with a monolayer, but the case of rose bengal leaves no doubt at all. This photodynamic lysin will produce hemolysis in a concentration of  $10^{-8}$  M in the light (Blum, 1935); taking an *in vitro* hemolytic system with  $2 \cdot (10^8)$  cells in a volume of 10 cc. of  $10^{-8}$  M rose bengal, we have a total surface of about  $2 \cdot (10^{10}) \mu^2$ , and about  $3 \cdot (10^3)$  molecules per  $\mu^2$ . If the area covered by each dye molecule is 1,000 Å<sup>2</sup>, only about 1/30th of the cell surface would be covered by the dye, even if all of it were concentrated at the cell surface, which it is not.<sup>5</sup> Calculating similarly for erythrosin, which produces lysis in a concentration of  $10^{-7}$  M in the light, about 1/3rd of the cell surface might be covered, but again this is a maximum estimate. The same conclusion is arrived at by considering the

<sup>5</sup> The shape of the rose bengal molecule when at the red cell surface is not known. Its molecular weight is about 1000; remembering that the serum albumin molecule, with a molecular weight of about 67,000 and some asymmetry, has a surface of about 9,000 Å<sup>2</sup>, 1000 Å<sup>2</sup> is probably an over-estimate of the surface of the dye molecule. It may be mentioned here that there is some legitimate objection to the use of rose bengal as an instance of a lysin which does not cover the cell surface, for the lysis is due, strictly speaking, to an oxidative process induced by the light to which the system containing the dye is exposed.

acceleration of saponin hemolysis, for benzene and *p*-di-brom-benzene produce considerable acceleration when there is only enough present to cover a part of the cell surface, even if all the available molecules are concentrated there (Ponder, 1939).

Because lysis occurs at spots or patches in the membrane, and because pigment escapes through holes, it does not follow, however, that the lysin acts only at these spots or patches. On the contrary, we suppose that it acts all over the membrane provided that it is present in sufficient concentration to do so, and combines with a membrane component or components in order to produce breakdown (Ponder, 1926 *a*; Ponder and Yeager, 1930). If we subscribe to the all-or-none law in relation to hemolysis (Saslow, 1929; Parpart, 1931), the first question is whether there is enough lysin in any given system

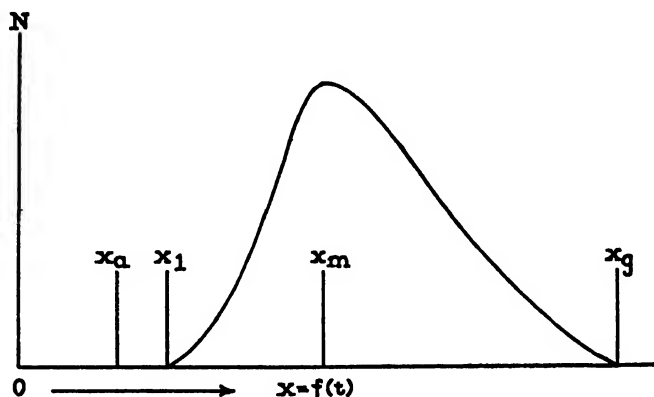


FIG. 3. Diagrammatic frequency distribution for resistances of various areas in the cell membrane. For explanation, see text.

to break down even one spot. In the case of any one red cell, we can suppose that the membrane is made up of areas of different degrees of resistance to the lysin, and that the resistance of these areas is distributed according to some form of frequency curve. This resistance is measured by the amount of lysin  $x$  required to break down the area, and, for simplicity's sake, may be thought of as the thickness of the membrane at the area under consideration, the thickness varying from area to area. There will then be a few areas of least resistance  $x_l$ , many of mean resistance  $x_m$ , and a few of greatest resistance  $x_g$ , but the resistance  $x_l$  is not zero, so the frequency distribution will be something like that in Fig. 3. The lytic reaction, or the "fundamental process," is a function of time,

$$dx/dt = kS(c - x)^n \quad (6)$$

so a time  $t$  must elapse before the quantity of lysin  $x_l$  is transformed as a result of its reaction with the membrane component, and before the weakest areas

break down. If hemolysis of the single cell is all-or-none, lysis will begin at this time, pigment escaping at such a rate as to give a fading time  $f$ . Meanwhile, even during this brief fading time, the fundamental process is proceeding all over the surface, next involving areas a little more resistant than  $x_i$ , then areas more and more resistant, and, if the concentration  $c$  of lysin is great enough and the fundamental reaction fast enough, destroying as much as 10 per cent of the membrane component within the fading time itself (Ponder and Marsland, 1935).<sup>6</sup> After the cell has hemolyzed the reaction continues by the lysin combining with the material of the ghost, to cease only when  $x_g$  has been transformed or the lysin exhausted.<sup>7</sup> In general, the number of holes  $N$  produced at any time  $t$  can be found by solving expression (6) simultaneously with

$$N = N_0 \int_0^x F(x) \cdot dx \quad (7)$$

$F(x)$  being the frequency distribution referred to  $x = 0$ , and not to  $x_m$ , as origin.

In any experimental hemolytic system, of course, the lysin is reacting with other cells at the same time. Each of these has areas of least resistance  $x_i$ , of mean resistance  $x_m$ , and of greatest resistance  $x_g$ , but, since the individual cells have different resistances to the lysin, the various values of  $x_i$  are themselves distributed according to some form of frequency distribution, and the same for all the values of  $x_m$ ,  $x_g$ , etc. It is these frequency distributions which are involved in the calculation of percentage hemolysis curves by the simultaneous solution of equations exactly like (6) and (7), the one for the fundamental reaction rate, and the other for the distribution of resistances (Ponder and Yeager, 1930, equations 3 and 5).<sup>8</sup>

<sup>6</sup> Because a cell can hemolyze in a system of low lysin concentration when only about 1/10,000 of its surface area is broken down to form a few small holes, it must not be supposed that such a small amount of destruction occurs when the amount of lysin is great. This point seems to give rise to some difficulty, as also does the idea of a lysin acting over the entire surface but producing lysis only at spots. The rusting of a tin can provides a very useful analogy.

<sup>7</sup> The reaction with the ghosts proceeds with a falling velocity constant and the equilibrium conditions are peculiar (Ponder, 1935), but these complexities do not affect the present argument.

<sup>8</sup> Historically, the idea of a frequency distribution of the resistances of areas in the individual cell membrane is only a further extension of the idea of discontinuity in hemolytic systems. In the first quantitative treatment of the kinetics of hemolysis, Arrhenius treated the cells as if they were molecules, and wrote the velocity of the lytic reaction as a function of the number of cells remaining unhemolyzed. This is all the more strange, as Arrhenius emphasized the importance of variation in cell resistance in several other connections. The idea of variation in resistance and of a fundamental reaction between the lysin and the cell surface was first clearly incorporated into the kinetics by Brooks (1919), and later developed by me.

If the lysin is present in concentration less than  $c_{\infty}$ , even the weakest areas do not break down, for  $x_1$  is not transformed. Further suppose that the cell surface is incompletely covered; e.g., that only 1/10th of it is covered. This will make no difference to the application of expression (7), for an area as great as 1/10th is certainly an almost perfect sample of the whole,<sup>9</sup> but the concentration of lysin will be reduced to 1/10th, and this will affect both the rate and the position of the asymptote in expression (6) by changing the value of the term  $(c - x)$ .

If an accelerator is present under such circumstances, its effect will be to increase the activity of the lysin by  $1/R$ , so that a sub-lytic concentration becomes lytic if it is greater than the value given by expression (4). But, since the surface is incompletely covered, the effect of the accelerator is reduced in proportion because it cannot affect a lysin which is not there to be affected; otherwise the reaction proceeds as before.

The falling-off of the acceleration in systems containing less lysin than  $c_{\infty}$  accordingly leads to the idea of hemolytic systems in which the cell surface is incompletely covered with lysin, while at the same time we undoubtedly have many cases (e.g. eosin, sodium stearate, smilacein, and, indeed, the majority of the less powerful lysins) in which the smallest amount of lysin needed for hemolysis is enough to cover the cell surfaces many hundred times. At this point we have to return to the beginning of the argument and define what we have been speaking of as "covering." The idea of monolayer formation will not do, so, remembering that the argument has been based on "complete covering" corresponding to  $c_{\infty}$ , and now dropping the idea of monolayer formation, we can substitute for "covering" *the production of a state resulting in the transformation of the quantity  $x_1$* . This definition does not involve monolayers or multilayers, but has reference to the reaction between the lysin and the membrane component and to that only. If the state corresponding to  $x_1$  is reached by the lysin in concentration  $c_s$  or greater and in the absence of an accelerator, lysis will take place (see Fig. 3), while if an accelerator is present, all the reacting lysin will be affected by it. If the lysin is less than  $c_{\infty}$ ,  $x_1$  will not be transformed, but the smaller amount which is transformed may be enough to produce lysis in the presence of an accelerator,<sup>7</sup> and the smallest amount which can be effective in this way, and which we can call  $x_a$ , is given by expression (4). Under such circumstances the full effect of the accelerator is reduced because the reactive lysin is less than  $c_{\infty}$ .

<sup>9</sup> If there is only enough lysin to cover 1/10th of the surface, it will not do to suppose that it covers 1/10th of the surface in one great patch (as in Ponder and Neurath's experiments), for the experimental results are not derivable from such a state of affairs by the reasoning which I have used. The necessary condition is one of discontinuity, in the sense that areas at which the lysin acts are separated by areas at which it does not.



This statement of the conditions covers the numerous cases in which the smallest amount of lysin capable of producing lysis is much greater than that required to cover the cell surfaces, the rarer cases in which it is much smaller, and those cases where, apparently coincidentally, the cell surface is just about covered. I think that too much emphasis has been placed on the last of these, perhaps because they can be so easily accounted for in the light of spreading experiments. The type of case which is more general, however, is the one first mentioned.

Finally, on this hypothesis we are able to arrive at some idea of the form of the frequency distribution  $F(x)$  in expression (7); *i.e.*, of the distribution of the resistances of the areas in the cell membrane. This we can do from Ponder

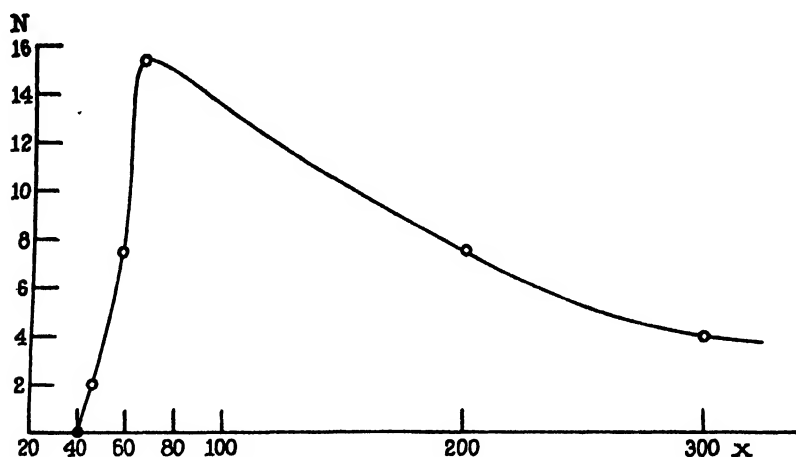


FIG. 4. Frequency distribution of resistances derived from Ponder and Marsland's values for fading times in saponin systems. Ordinate, number of areas of a resistance  $x$ , in arbitrary units; abscissa, lysin  $x$  transformed, also in arbitrary units. For further description, see text.

and Marsland's Fig. 1, which shows the average fading time of cells selected at random as a function of the dilution of lysin in the system. The curve is sigmoid, and this ought to have suggested to us at the time that it involves the integral of some frequency distribution. Plotting the fading time as ordinate and the lysin concentration in micrograms/cc. as abscissa gives another sigmoid curve, which, when differentiated graphically, gives Fig. 4. This curve is an approximation to the form of the desired frequency distribution  $N = F(x)$ . It is very skew. This is no doubt due in large part to both observation and theory being faulty for concentrations greater than about 100 micrograms/cc.<sup>10</sup>

<sup>10</sup> Two assumptions which are made are (a) that the diameter of each of the  $N$  holes is uniform, and (b) that the amount of lysin transformed during the fading time is

(a dilution of 1 in 10,000), but it may be a reflection of the fact that the amount of lysin used up at equilibrium in a system in which stromatolysis is allowed to proceed is very much greater than that needed to complete hemolysis (Ponder, 1935). If so, we have another instance of extreme skewness in connection with hemolytic systems, for the distributions of the resistances of individual cells to saponin are very skew (Ponder, 1930); here again, however, there is doubt as to the underlying theory and as to whether correction terms are not required (Ponder, 1931, 1934 *a*<sup>11</sup> and 1934 *b*<sup>12</sup>). We can, however, make one deduction from the shape of the curve. The origin  $N = 0$  occurs, on the scale used, at about 40 micrograms, while the mode occurs at about 80 micrograms. Thus the amount of lysin transformed as we pass from  $x_i$  to  $x_m$  is about the same as that transformed during the "latent period;" i.e., as we pass from zero to  $x_i$ . Further, the scatter  $(x_m - x_i)/x_m$  is only about 0.5, which means that the resistance of the membrane, while varying from point to point, is remarkably uniform. This is at least in keeping with what is known about the considerable degree of regularity of membrane ultra-structure (Schmitt, Bear, and Ponder, 1936, 1938).

#### SUMMARY

The acceleration of saponin hemolysis by benzene, indol, and nonyl alcohol has been investigated as a function of temperature, and it has been found that these accelerators have negative temperature coefficients. This points to their being concentrated at the cell surface, and to the surface being the seat of their accelerating action.

It is shown that the accelerating power of indol (used as a typical accelerator) is constant so long as the lysin in the system is capable of producing lysis *per se*, but that the acceleration falls off when only sublytic concentrations are present. The relations are expressed in a series of equations, and explained in terms of the reactions among the accelerator, the lysin, and the membrane component,

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proportional to the lysin concentration in the system. Both assumptions are good enough for a first approximation, and, after all, the curve in Fig. 4 is little better than the result of curve tracing. The course of the curve above the mode is, I think, of very little significance, for neither the theory nor the observations are good enough. The apparent skewness depends a great deal on the length of the very short fading times in systems containing concentrated lysin, and a small change in the course of Ponder and Marsland's curve would produce an enormous change in the course of the upper part of Fig. 4. Under any circumstances, moreover, the form of the frequency distribution above the mode is of little interest in connection with hemolytic systems, for the lysis of any individual cell commences when  $x_i$  is transformed and, except in the case of the most rapid reactions, must be complete by the time  $x_m$  is reached.

<sup>11</sup> P. 193 and following pages.

<sup>12</sup> P. 291.

which breaks down in spots, rather than uniformly, when lysis occurs. The argument involves a consideration of the idea that a monolayer of lysin at the cell surface is necessary for hemolysis, of Abramson's hypothesis of "key spots" on the surface, of the rate of escape of hemoglobin from the hemolyzing cell, and of the results of electrophoretic and impedance measurements. The existing theory of the kinetics is extended by introducing the idea of a variation in resistance from point to point in the cell membrane; in this form it describes the situation so far as is at present known, and shows that the results of the various methods of investigation are consistent with each other. The only idea discussed which seems to have little foundation is that lysis is determined by the formation of a monolayer of lysin at the cell surface; when this occurs, it must do so only as a special case. Finally, a semi-quantitative description of the frequency distribution of the resistances in the membrane is derived from existing data. The variation in resistance which it is necessary to assume is quite small, as might be expected in the case of a membrane with a regular ultra-structure.

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# ANALYTICAL DIFFUSION OF INFLUENZA VIRUS AND MOUSE ENCEPHALOMYELITIS VIRUS

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In a recent publication, (1) a method was described for the analytical study of the diffusion of biologically active material. The present paper reports an attempt to apply this method to the study of two animal viruses, epidemic influenza A and mouse encephalomyelitis.<sup>1</sup>

## *Method*

In brief the technique (1) consists in placing a layer of solvent above a layer of solution containing the virus, allowing the latter to diffuse upwards for a few days, sampling at various heights, and determining the virus titer in the samples. The original virus concentration, expressed in lethal doses, or minimal infective doses, per inoculum, is  $C_0$ , and  $C_x$  is the concentration  $x$  centimeters above the initial boundary, at the end of the experiment. In order to determine the diffusion constant  $D$ ,  $\log C_x/C_0$  is plotted against  $x$ , and the theoretical curve is drawn which gives the most satisfactory fit. For  $x = 0$ ,  $\log C_x/C_0$  is always equal to  $-0.3$ . (This point, therefore, does not have to be determined by experiment; it is indicated on the charts as a half circle.) The diameter  $d$  of the virus particles may then be derived from the diffusion constant, the assumption being made that the diffusing particles are approximately spherical.

In the experiments reported here, the virus concentration at the bottom of the diffusion cell was taken as  $C_0$ . Sampling was done up to 1.2 or 1.3 cm. above the initial boundary; above this level the fluid usually still contained a trace of virus, but too little for quantitative titration.

## *Diffusion of Influenza A Virus from Mouse Lung*

### *Material and Titration*

The stock material was a suspension of mouse lung tissue infected with the PR8 strain, prepared by grinding infected lungs with aluminum and salt solution (usually 0.15 M NaCl or 0.04 M Na borate) so as to make a 10 per cent suspension, centrifuging at 2500 R.P.M. for 30 minutes, and discarding the sediment. This yielded a reddish turbid fluid, containing about 1 per cent protein in solution; much

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<sup>1</sup> Part of the experiments reported in this paper were performed at the laboratories of the International Health Division of The Rockefeller Foundation, New York.

of the latter was hemoglobin and blood serum proteins; some was lung tissue protein, characterized by irreversible precipitation below pH 6. The limiting lethal dilution of the suspension for mice was about  $10^{-8}$ , when inoculated intranasally in 0.05 cc. amounts. In one experiment, (No. 5) the material used was more concentrated: The suspension was made of 10 gm. of lungs ground with 4 cc. of mouse serum and 6 cc. of 0.85 per cent NaCl. The titer was about  $10^{-8}$ . The drop in titer of these preparations at 4° was about 0.3 log after 2 to 4 days.

For titration, serial decimal dilutions of the diffusion samples were made in 10 per cent horse serum broth; 4 to 6 young albino mice were inoculated intranasally, under ether anesthesia, with 0.05 cc. of each dilution. The 50 per cent mortality end-point was calculated by the method of Reed and Muench (2) and based on the

TABLE I  
*Diffusion of Influenza A Virus from Mouse Lung*

Exp. No.	Diffusing solution						Diffused into	Diffusion time
	Buffer			Total protein concentration	Control virus titer M.L.D.			
	Composition	Molarity	pH					
				per cent	log		sec.	
1	0.12 M NaCl; 0.02 M Na borate	0.14	9.5	1	5.3	Water	188,000	
2	0.55 M NaCl; 0.02 M Na borate	0.57	9.5	1	5.5	Water	153,000	
3	0.04 M NaCl; 0.02 M Na borate	0.06	9.7	1	4.5	Same buffer	243,000	
4	0.025 M Na phosphate; 0.02 M Na borate	0.045	8.8	1	4.7	Normal lung solution in same buffer	430,000	
5	0.15 M NaCl	0.15	7.4	6	6.0	Water	256,000	

number of dead and surviving mice 10 days after inoculation; the virus concentrations were expressed in lethal units per 0.05 cc.

#### EXPERIMENTAL

In order to determine whether the results obtained were in any way dependent on the conditions of diffusion, the latter were varied as much as possible. They have been summarized in Table I.

The approximate composition of the buffers is given in the second column; they were all alkaline, since the virus is less stable in acid medium. In Experiment 1, a small amount of stock borate buffer (pH about 9.8) was added to the original lung suspension; in Experiment 2, borate buffer and solid NaCl were added; in Experiment 3, the material was first dialyzed against the buffer used, before diffusion. Experiment 4 was prepared as follows: 10 cc. of infected lung suspension in 0.04 M borate was dialyzed for 3 days in the cold against 10 cc. of normal mouse lung suspension in 0.05 M Na phosphate; the

resulting pH was 8.8. In this manner the salt composition and salt concentration must have become the same in both solutions; furthermore the protein concentration too must have become identical on both sides, due to the osmotic pressure; it is also probable that the protein composition was about the same in the two fluids. The coarser particles were removed from the normal lung suspension by filtration through asbestos, whereas the infected suspension was left unfiltered. Great care was used in dialysis to avoid contamination of the normal fluid, which was the fluid into which diffusion was to take place. Finally, for Experiment 5, a more concentrated virus preparation was used, as described above.

Thus, the salt molarity of the diffusing solutions ranged in these experiments from 0.045 to 0.57; the pH from 7.4 to 9.7. The total protein concentration was in general 1 per cent, in one case 6 per cent. Diffusion was allowed to take place into pure water (Experiments 1, 2, and 5); into buffer (Experiment 3); into buffered normal lung suspension (Experiment 4). Diffusion time was varied from 2 to 5 days.

## RESULTS

The results have been plotted in Fig. 1. The ordinates give the difference between the titer of the control (the titer at the bottom of the diffusion cell) and the titer at various levels above the initial boundary; the abscissae measure the distance above the initial boundary.

Examination of Fig. 1 reveals two facts: One is that the results from all five experiments were quite similar in spite of great differences in the conditions of diffusion; the other, that the values obtained obviously did not yield ideal diffusion curves (which should have been convex toward the upper right hand corner of the chart), but broken curves composed of two fairly distinct limbs. Since the results appeared to be completely independent of such experimental conditions as salt concentration, pH, presence or absence of a simultaneously diffusing salt or protein, one may be allowed to draw the conclusion that they were dependent only on the particle size of the virus in the preparation; and since they did not yield ideal diffusion curves, one may conclude that the virus was not homogeneous.

Whereas an attempt to fit the observed values with one curve would have been quite unsatisfactory, much better agreement was found when two curves were used, to express the simultaneous but independent diffusion of two sets of particles. This is shown in Fig. 1. The curves chosen for the purpose were: (A) a curve expressing the diffusion of particles  $200\text{ m}\mu$  in diameter (diffusion constant  $D = 0.13 \times 10^{-7}$ ), starting from the axis of ordinates at  $-\log C_x/C_0 = 0.3$ ; (B) a curve expressing the diffusion of particles  $6\text{ m}\mu$  in diameter ( $D = 4.3 \times 10^{-7}$ ) and fitting the values above  $x = 0.2$  approximately, thus intersecting the axis of ordinates about  $-2$  or  $-3$ .



The curve chosen to express the diffusion of the small fraction of the virus is thus displaced downwards by an amount equal to the proportion of the small fraction in the total virus. This is simply because the reference concentration  $C_0$  is that of the total virus and not that of the small fraction only. A correct representation of the simultaneous diffusion of two sets of particles would obviously be a single compound curve; but, as can be shown graphically, if the two sets are of very different sizes and in very different amounts, two curves drawn independently will closely resemble the correct compound curve. Indeed, it is

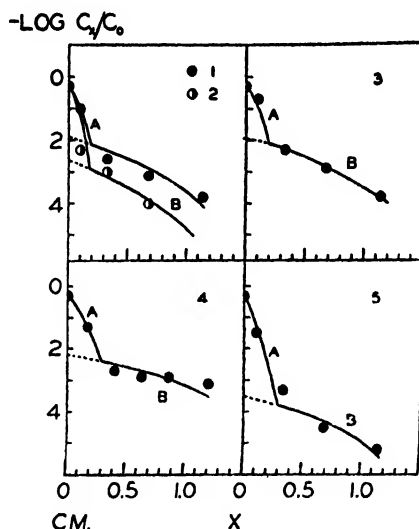


FIG. 1. Diffusion of influenza A virus. Temperature  $4^{\circ}$ . (A): Theoretical curves calculated for the diffusion of spherical particles  $200\text{ m}\mu$  in diameter (diffusion constant  $D = 0.13 \times 10^{-7}$ ). (B): Same for particles  $6\text{ m}\mu$  in diameter ( $D = 4.3 \times 10^{-7}$ ). Curves (B) have been displaced downwards so as to fit the points corresponding to the diffusion of the fast moving fraction. Diffusion time: Experiment 1 and 2, 2 days; Experiment 3 and 5, 3 days; Experiment 4, 5 days (cf. Table 1).

apparent that below  $x = 0.3$  approximately, there can be too little of the small fraction to affect appreciably curve (A), and, above  $x = 0.3$ , too little of the large fraction to affect appreciably curve (B).

Because of the inaccuracy of the analytical procedure it is impossible to say whether the distribution in two distinct fractions was as clearcut as has been assumed, and evidently the choice which was made of diameters of  $200$  and  $6\text{ m}\mu$  respectively can only be a rough approximation. But it is probable that the particle diameter of the large fraction must have been about 20 or 30 times that of the small fraction, which means a molecular weight ratio of the order of 10,000 to 1. In Experiments 1 to 4, since curve (B) intercepts the axis of ordinates about  $-2$ , the proportion of the small virus fraction in the total virus

must have been about 1:100; in Experiment 5, about 1:1000. As mentioned in the beginning, the lung suspension in Experiment 5 was made in mouse serum and was more concentrated, which may account for the difference.

Thus it appears that in the influenza preparation investigated about 1 per cent, or 0.1 per cent, of the total infectivity was present in particles about the size of normal serum albumin or globulin molecules. The rest of the virus, that is, the bulk of it, appeared to be present in particles of the order of magnitude which has been found by ultrafiltration or ultracentrifugation (3, 4), or about 100 or 200  $m\mu$ .

Other diffusion experiments with mouse lung virus (not reported) suggested that the fast diffusing fraction was less stable than the rest of the virus. Thus in one case the samples removed after diffusion, placed in celluloid tubes and forming a shallow layer at the bottom of the tubes, were kept 24 days at  $-76^{\circ}$  before titration. A general drop in titer was then found but much more marked in the samples coming from the upper part of the diffusion cell. The same phenomenon was observed when, before diffusion, the suspension was rocked for 2 hours in a shallow trough at  $28^{\circ}$ , exposed to light and air.

*Diffusion of Influenza A Virus from Chick Extra-Embryonic Fluids*  
*Material and Methods*

The material has been investigated by Henle and Chambers (5). It was obtained from 11 to 12 day old embryos infected with the virus.<sup>2</sup> If the normal embryo at this stage the volume of the allantoic fluid is about 6 cc., that of the amniotic fluid 2 cc. They contain about the same salts as a serum ultrafiltrate and are both nearly protein-free until the 12th day; the allantoic fluid receives from the embryo increasing amounts of uric acid. About the 12th day the amnion opens into the albumin sac, thus acquiring suddenly a high protein concentration (6). The infected fluids used were almost clear and their protein content was insignificant. We found that both normal and infected fluids contained an appreciable amount of small particles, the size of which was about at the limit of ordinary microscopic visibility (*ca.*  $0.2\mu$  in diameter) and which appeared to be identical with the mitochondria from normal chorioallantoic cells. A few red cells and cellular debris in the fluid were removed by low speed centrifugation. For each diffusion experiment a different batch of fluid was used.

Chambers and Henle (7) have made a detailed study of the behavior of this fluid in the angle centrifuge. They found that the titer of the virus in the supernatant after 30 minutes at 25,000 R.P.M. in the ultracentrifuge of Rawson, Scherp, and Lindquist (8) remained quite high (only 1 or 2 log below the original titer); they suggested that an appreciable fraction of the virus must have a

<sup>2</sup> We are much indebted to Dr. W. Henle and Dr. L. A. Chambers for supplying us with the material and for performing the titrations.

particle diameter of 10  $m\mu$  or less. For three of the diffusion experiments reported here the fluid was first diluted 1:1 with buffered saline (0.15 M NaCl, 0.01 M  $PO_4$ , pH 7.0), then ultracentrifuged as shown in Table II. In Experiment 4, the supernatant, after a first run at 25,000 R.P.M. for 30 minutes, was centrifuged again, and the second supernatant used for diffusion. The fluid from the top of the tubes was pipetted off with care each time, to avoid possible stirring of the sediment.

Before diffusion, enough NaCl solution was added to the fluid to make the total salt concentration about 2 per cent. Diffusion took place into distilled water, for 1 or 2 days. The technique was as described for mouse lung, except that the cell was placed in a mixture of ice and water at 0°, instead of a constant temperature bath at 4°.

TABLE II  
*Diffusion of Influenza A Virus from Chick Extra-Embryonic Fluids*

Exp. No.	Diffusion time	Virus titer of control M.I.D.	Remarks
	<i>sec.</i>	<i>log</i>	
1	153,000	5.8	Not centrifuged
2	87,000	4.9	Supernatant after ½ hr. centrifugation at 25,000 R.P.M.
3	86,400	3.8	" "
4	149,000	4.2	Supernatant recentrifuged ½ hr. at 25,000 R.P.M.

The titration of the diffusion samples was essentially as described for lung virus, except that the minimum infective dose (the dose just sufficient to induce one pulmonary lesion in a mouse) was chosen for the end-point. The M.I.D. titer thus obtained was about 2 log above the M.L.D. titer and was found more convenient since titrations could be made over a wider range of dilutions. The titer of the controls given in Table II was that of the virus at the bottom of the cell after diffusion. Except in Experiment 4, the material was not fresh and had been stored at -15° or -75° for from 1 to 5 weeks; this usually caused an appreciable drop in titer from that of the fresh material.

#### RESULTS

They are shown in Fig. 2. They do not differ essentially from the mouse lung experiments, since they all reveal the presence of a fast diffusing fraction, which a curve drawn for the theoretical diffusion of particles 6  $m\mu$  in diameter fits approximately. In Experiment 1, the values obtained did not warrant the drawing of two curves, as was done for mouse lung virus. In Experiment 2, ultracentrifugation did not achieve a clearcut separation of the fast diffusing

fraction, which, on theoretical grounds, might be expected only of a mixture containing two sets of particles of very different size. In Experiments 3 and 4, centrifugation was more effective, since the curves obtained are almost smooth curves; here the intercept of the curves with the y axis indicates that the ratio of the fast diffusing fraction to the rest of the virus must have been of the order of 1 to 3. Too little is still known of the nature of the particulate components of the fluid investigated, and of the state and stability of the virus, to warrant further speculation at this point.

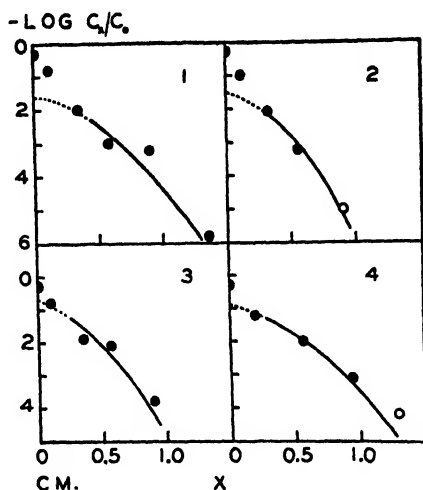


FIG. 2. Diffusion of influenza A virus from chick extra-embryonic fluid. Temperature  $0^{\circ}$ . Theoretical curves calculated for the diffusion of spherical particles  $6 \mu$  in diameter ( $D = 4.2 \times 10^{-7}$ ). Diffusion time: Experiment 1 and 4, 2 days; Experiment 2 and 3, 1 day. (The open circles represent titers which were obtained only as maximum values.)

An attempt to rediffuse the diffusate from a first experiment proved futile, the virus having completely lost its infectivity during the second diffusion. This confirmed the impression gathered from mouse lung preparations that the virus is particularly labile in its more disperse state.

It may be concluded from these experiments that influenza A virus in chick extra-embryonic fluids was in an inhomogeneous condition and that a small fraction of it appeared, from diffusion measurements, to have a particle diameter as small as  $6 \mu$ .

#### *Diffusion of Mouse Encephalomyelitis Virus*

The virus isolated by Theiler and recently studied in detail by Theiler and Gard (9) was used. It is fairly resistant and, thanks to Gard's method (10) it

can be titrated with sufficient accuracy. Ultrafiltration by Elford's method (4) gave Theiler and Gard a value of 9–13  $m\mu$  for the diameter of the virus particles.

### *Material and Titration*

A 20 per cent suspension in pneumococcus broth of ground mouse brains infected with strain GD VII was prepared; it was centrifuged at 2500 R.P.M. for a short time and the sediment discarded. Titration of the diffusion samples was carried out as described by Gard (10), using the intracerebral inoculation route.

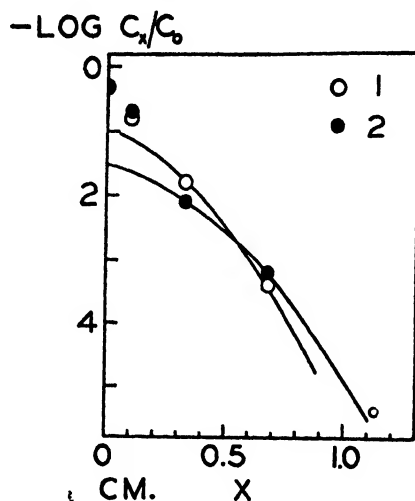


FIG. 3. Diffusion of mouse encephalomyelitis virus. Temperature 4°. The curves express the theoretical diffusion of spherical particles 15  $m\mu$  in diameter ( $D = 1.7 \times 10^{-7}$ ). Diffusion time: Experiment 1, 158,000 seconds, Experiment 2, 237,000 seconds.

The values obtained by this titration method could be read directly as the logarithms of the relative concentrations of the diffusion samples.<sup>3</sup>

### RESULTS

In Experiment 1, the 20 per cent brain suspension in broth was directly diffused against water for 2 days (158,000 sec.). In Experiment 2, the suspension was first dialyzed against 0.16 M NaCl and Na phosphate buffer, pH 7.3, then diffused against another portion of the same buffer for 3 days (237,000 sec.). Fig. 3 shows that the results from both experiments are closely

<sup>3</sup> We are much indebted to Dr. M. Theiler for supplying us with the material and for performing the inoculations.

comparable. In the sample withdrawn at  $x = 1.14$  cm. no virus was detected; the small circle drawn on the chart at this point gives the minimum relative concentration at which virus would have been detectable by Gard's method; in other words, any theoretical curve drawn to fit the other points should pass below approximately  $5.5 \log$  at  $x = 1.14$ .

The same remarks made about influenza apply to these results as regards the choice of curves. Evidently the virus was not homogeneous, and an attempt to fit all the points with one curve would have been unsatisfactory; on the other hand, the results were not such as to warrant the drawing of two separate curves, as was done with mouse lung influenza. One can nonetheless obtain an idea of the diameter which must have been that of at least some of the particles by drawing a curve through the values for  $x = 0.33$  and  $x = 0.68$ . This has been done on the chart, and yields a diameter of  $15 \mu$ ; the intercept of the curve with the axis of ordinates indicates that this fraction represented roughly 10 per cent of the total virus. The value found by diffusion for the particle diameter of an appreciable fraction of the virus is therefore in fair agreement with ultrafiltration data.

#### DISCUSSION

The results reported above from mouse encephalomyelitis virus diffusion require little comment, since they agreed rather satisfactorily with ultrafiltration data; they suggested, however, that the preparation was not homogeneous, and that only about 10 per cent of the activity was present in particles  $15 \mu$  in diameter; whether these were the smallest units could not be ascertained; it appeared only that smaller virus units could not have been present in considerable quantity.

The experiments with influenza virus revealed the presence of a fast diffusing fraction which the methods thus far employed had not been able to detect. Experience gained from the diffusion of catalase (1), and the fact that great variations in the conditions of diffusion did not influence the type of curves obtained, warranted the conclusion that the results were not due to experimental error. The persistence of infectivity in the supernatant after ultracentrifugation of extra-embryonic fluid offered additional evidence that part of the virus must have existed in a highly disperse state.

The apparent contradiction between our value of  $6 \mu$  and that of about  $100 \mu$  derived from ultrafiltration and ultracentrifugation of mouse lung virus by Elford and collaborators (3, 4) can be easily explained if one bears in mind that the fast diffusing fraction of our preparations represented only a small percentage of the total, and therefore could be detected only by a method of adequate sensitivity. Ultrafiltration and ultracentrifugation, as well as ultramicroscopy, as they have been applied thus far to the study of animal viruses, can be expected to give information only on the state in which the bulk

of virus infectivity is present. Thus when mouse lung virus is filtered it appears now that the end-point obtained is determined by the size of the larger particles, the diffusion of which gave, in our experiments, the first part of the curves shown in Fig. 1; it should not seem surprising that a small, highly disperse, and probably easily adsorbed fraction of the virus should not be able to pass a membrane filter to an appreciable degree.

If the ultimate virus units are comparatively small molecules, the larger particles become relatively unimportant. Similar particles have been observed in preparations of numerous viruses, and because they carry most of the infectivity they have usually been assumed to represent the actual virus units. But in recent years the specificity of these particles has become very doubtful. Claude (11) first observed that some normal tissue preparations contained non-active particles which in size and chemical composition were exactly similar to the supposed agent of chicken tumor I. Other investigations have since been carried out by numerous authors (reviewed by Henle and Chambers (12)) on material from various sources, leading to the general conclusion that particles 0.1 to 0.3 $\mu$  in diameter, or material sedimentable only by ultracentrifugation, can be obtained from a great variety of normal tissues. Henle and Chambers (12) have recently observed that such particles exist in preparations of normal mouse lungs. We have found, as mentioned above, that particles of about the same size are present in the extra-embryonic fluids of the chick, both normal and infected, and appear to be identical with the mitochondria of normal cells. It now seems probable that in influenza preparations such particles or mitochondria act as virus carriers, the actual virus units being of a much smaller order of magnitude. Preliminary diffusion experiments have suggested to us that this is also true of vaccinia. Bronfenbrenner and collaborators (13) have reached the same conclusion on the nature of bacteriophage. The ultimate units of these viruses appear to consist of comparatively small molecules adsorbed on non-specific colloidal carriers.

#### SUMMARY

Analytical diffusion has been applied to a study of influenza A virus in mouse lung, influenza A virus in the extra-embryonic fluids of the chick, and mouse encephalomyelitis virus in mouse brain.

The results from influenza in mouse lung suggested that about 99 per cent of the infectivity was present in particles 200  $m\mu$  in diameter, and 1 per cent in particles 6  $m\mu$  in diameter.

The results from influenza in extra-embryonic fluids indicated that the preparation was inhomogeneous and that the smallest virus units were about 6  $m\mu$  in diameter.

The results from mouse encephalomyelitis virus indicated that the prepara-

tion was also inhomogeneous, with 10 per cent of the infectivity in particles about 15  $\mu$  in diameter.

It has been suggested that in virus preparations normal colloidal particles can act as carriers of much smaller virus units.

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## P<sub>32</sub> UPTAKE BY NUCLEI

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As early as 1856, Francis Gurney Smith described and illustrated free nuclei obtained from skin and tumor tissue by the action of acetic acid (1), but curiously enough no use was made of this observation in subsequent biochemical studies of nuclei. From pus cells treated with artificial gastric juice, Miescher (2) isolated "nucleins," substances presumably characteristic of nuclei. Ploz (3) later showed that the insoluble residues of hemolyzed avian red blood cells gave characteristic "nuclein" reactions, and in 1904 Ackermann (4) determined the phosphorus and nitrogen content of nuclei obtained by this method. Eighty years after Smith's description of free nuclei, Crossmon (5) showed that small quantities of nuclei, sufficient for microscopic examination,

could be isolated by teasing cardiac muscle in 5 per cent citric acid. Stoneburg (6) studied the lipid content of nuclei isolated from muscle and tumors by the combined action of citric acid and pepsin. He found it impossible to isolate nuclei from liver by this technique. It has been found possible to isolate nuclei from the liver and tumors of mice and rats by the action of 5 per cent citric acid alone. Studies of the rates at which P<sub>32</sub> is bound by nuclei and cytoplasm, using this technique for isolating nuclei are described in the following experiments.

### *Materials and Methods*

Mice (20–25 gm.) of the Strong A strain were used as hosts for the Lawrence-Gardner lymphoma (7), and mice of the Swiss strain for sarcoma 180. Inbred rats of the Slonaker strain were used for the carcinoma 256. Injections of P<sub>32</sub> were timed so that the mouse tumors were 14–15 days old when removed. The rat tumors were 3–4 weeks old when used. The mice were given 0.1 cc. of isotonic Na<sub>2</sub>HPO<sub>4</sub> containing 5–11 microcuries of P<sub>32</sub> by intravenous injection. In the experiments with the mice 10 animals were used in each run.

Animals were anesthetized with nembutal and the livers perfused with saline through the hepatic vein until cleared of blood. Livers and tumors were then rinsed in ice cold saline, transferred to 5 volumes of cold 5 per cent citric acid in which the tissues were cut to small pieces with scissors, and allowed to stand for 30 minutes at

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2-5°C. They were then ground with mortar and pestle and passed through 2 layers of cheese-cloth. The suspension so obtained was centrifuged at low speed for 2 minutes to remove tissue fragments, and then at 2400 R.P.M. for 10 minutes. The nuclei were found in the bottom layer, the cytoplasmic fragments in the supernatant and upper solid layers. Nuclei with cytoplasmic fragments still attached were in the zone between the clean nuclei and the cytoplasm. In the liver, the latter was easily identified macroscopically since it had a brown color. The supernatant and upper solid layers were removed, the nuclei resuspended in 5 per cent citric acid and again centrifuged at 2,400 R.P.M. for 1-2 minutes. This process was repeated, usually 4-6 times, until very few cytoplasmic fragments were found in the upper solid layer, and the supernatant was no longer cloudy. The mass of washed nuclei so obtained was white. The same procedure was used with tumor tissue. In this case the cytoplasm had a grayish translucent appearance and since it was not as easily identified macroscopically each separation was examined with the microscope. The suspension of nuclei was then brought to a fixed volume with 5 per cent citric acid, 1 or 2 cc. pipetted into an ashing capsule for determination of radioactivity, and another fraction set aside for extraction. In each run a small portion of the suspension was used to determine the number of nuclei and the number and size of any non-nuclear fragments per cubic centimeter of suspension by hemocytometer count and measurement with an ocular micrometer. The volume of contaminating cytoplasmic fragments was found to be less than 0.5 per cent of the nuclear volume in all experiments. Another portion was centrifuged for exactly 30 minutes at 2400 R.P.M. in Bauer and Shenck (8) tubes to determine the relative volume of solid and fluid. If the centrifugation was prolonged beyond 30 minutes the nuclei became angular (usually hexagonal) in outline.

Small portions of tissues of all animals from which nuclei were to be isolated were put in weighing bottles and weighed as quickly as possible after removal. They were then transferred to ashing capsules and dried. The suspension of nuclei and an aliquot of the injected solution were also evaporated to dryness in ashing capsules. All activity measurements were made with an ionization chamber using a General Electric Co. FP-54 electrometer tube in a DuBridge circuit (9) and checked against a uranium standard.

To determine whether the liberation of nuclei was due to some specific action of the citrate ion or to the hydrogen ion concentration the following experiments were performed. Tissues were treated as previously described, substituting 5 per cent acetic acid and 5 per cent boric acid in place of citric. Nuclei could not be separated after treatment with boric acid but after treatment with acetic acid the nuclei could be isolated by centrifugation as readily as after citric. Liver pulped in 0.9 per cent NaCl showed on microscopic examination that the nuclei were completely free from the cytoplasm but were homogeneous rather than granular in appearance; *i.e.*, they apparently were not coagulated. When treated the same way in isotonic MacIlvaine buffer at pH 7.0, the nuclei did not separate from the cytoplasm. When pulped in buffer at pH 5.0, the nuclei were free with occasional small tabs of adherent cytoplasm, and had a granular appearance. At pH 6.0 the nuclei were free but did not appear granular until the tissue was immersed in the buffer for 1½ hours. Evidently the separation of the nucleus from the cytoplasm and its coagulation are due to an increase in hydrogen ion concentration and not to the action of a particular anion. In the case

of tissue ground in 0.9 per cent NaCl there is apparently sufficient acid liberated (the acid of injury of the microdissectionists) to free the nucleus from the cytoplasm, but not enough to produce coagulation.

## RESULTS

### *Chemical Exchange vs. Metabolism*

To determine whether the  $P_{32}$  was taken up by the nuclei by simple chemical exchange, the following experiments were performed: Nuclei were isolated from the livers of 10 mice and suspended in 3 cc. of a solution containing 15 mg.  $Na_2HPO_4$  /cc. with a total activity of 157.2  $\mu$ c. After shaking for 1 hour the nuclei were washed 4 times with 5 per cent citric acid. With each washing, the activity recovered was 145.2, 8.60, 0.900, and 0.215  $\mu$ c respectively. The nuclei contained 0.429  $\mu$ c and since the volume of nuclei used was 0.695 cc., the activity per cubic centimeter of nuclei was 0.617  $\mu$ c or 0.39 per cent of the activity originally contained in the solution. In a similar experiment the original solution contained 62.8  $\mu$ c in 4 cc. solution. The nuclei were washed 7 times. The washings contained 57.0, 5.41, 0.290, 0.044, 0.009, 0.006, 0.004  $\mu$ c respectively. The washed nuclei had an activity of 0.248  $\mu$ c/cc. or 0.395 per cent of the total added activity. In a third experiment with 87.0  $\mu$ c the nuclei retained 0.20 per cent of the activity in the solution after 7 washings. Isolated nuclei, therefore, will not bind more than 0.2-0.4 per cent of the phosphorus of an isotonic solution of  $Na_2HPO_4$  in which they are immersed. Since clumping of nuclei occurs when the phosphate solution is added to the nuclei suspended in citric acid, a considerable portion of the  $P_{32}$  found associated with the nuclei may simply be adsorbed. If this were the case, presenting other surfaces on which the phosphate could be adsorbed should decrease the amount apparently bound by the nuclei. This is shown to be the case in the following experiment.

The livers of 10 mice were cleared of blood by perfusion, cut into thin slices, and shaken for one-half hour at room temperature in a 10 cc. solution containing 8 cc. of 0.9 per cent NaCl and 2 cc. of 1.5 per cent  $Na_2HPO_4$  containing 206  $\mu$ c. When the nuclei were removed and washed 7 times in citric acid they were found to have an activity of 0.023  $\mu$ c/cc.; i.e., 0.011 per cent of the total added activity. In the *in vivo* experiments where 5-10  $\mu$ c of  $P_{32}$  was injected into the circulatory system the liver nuclei were found to contain 3 per cent of the added activity or about 300 times as much as in the case of the liver slices shaken with  $P_{32}$ . It follows from these and the previous experiments that the uptake of  $P_{32}$  from the blood stream cannot be accounted for by exchange but must be attributed to the metabolic activities of the cells involved.

### *Rate of $P_{32}$ Uptake*

The  $P_{32}$  retained by the liver and lymphoma tissue at various times after injection is shown in Fig. 1 and Table I. In the liver the maximum  $P_{32}$  content (9.5 per cent) is observed at 1 hour after injection as early as determinations

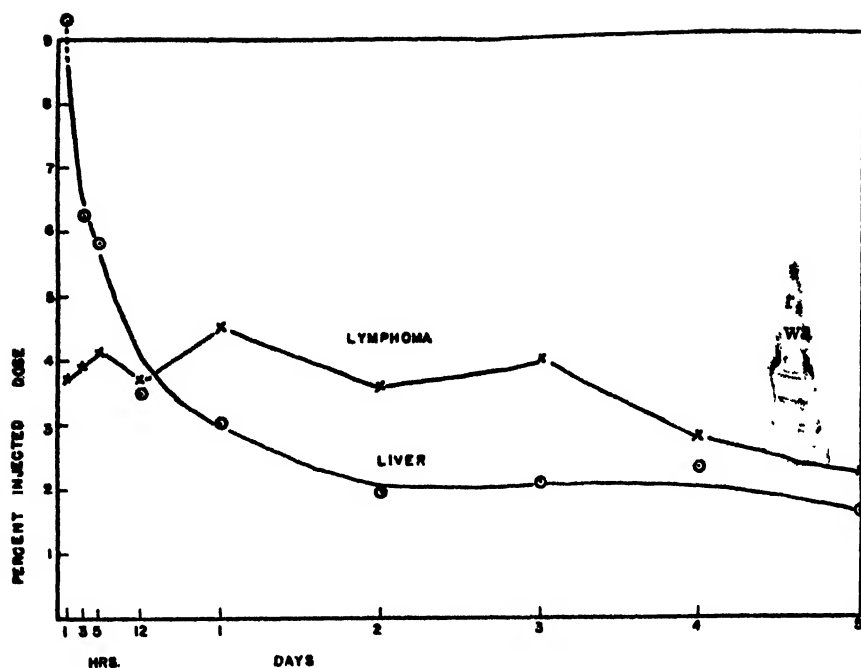
FIG. 1.  $P_{32}$  uptake by tissues

TABLE I  
*Lymphoma*  
 Activity As Per Cent Injected Dose

Time after injection	Nuclei		Tissue		Nuclei/tissue	
	Liver	Tumor	Liver	Tumor	Liver	Tumor
1 hr.	2.995	1.255	9.57	3.72	0.311	0.337
3 "	1.76	2.57	6.24	3.90	0.286	0.689
5 "	1.66	4.85	5.825	4.155	0.284	1.13
12 "	1.25	6.41	3.45	3.70	0.371	1.37
1 day	1.23	6.395	3.035	4.11	0.412	1.59
2 "	0.945	7.17	1.96	4.06	0.398	1.78
3 "	0.97	7.02	2.12	3.68	0.461	1.91
4 "	1.03	6.09	2.34	2.80	0.449	2.19
5 "	0.71	5.47	1.64	2.27	0.438	2.35

The value at each time interval is the mean of 2-4 determinations with 10 mice. The activity of the nuclei is given as per cent/cm.<sup>3</sup>, and that of the tissue as per cent/gm.

could be made, and is followed by a very rapid decrease to an asymptotic level at about 2 per cent. The lymphoma on the other hand shows a practically constant level at 4 per cent from 1 hour to 3 days after injection, followed by a

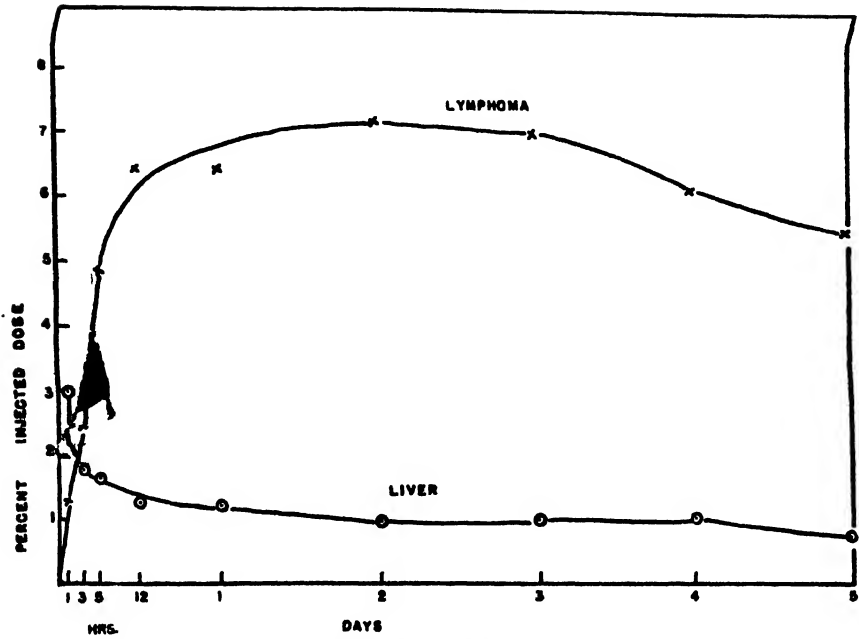
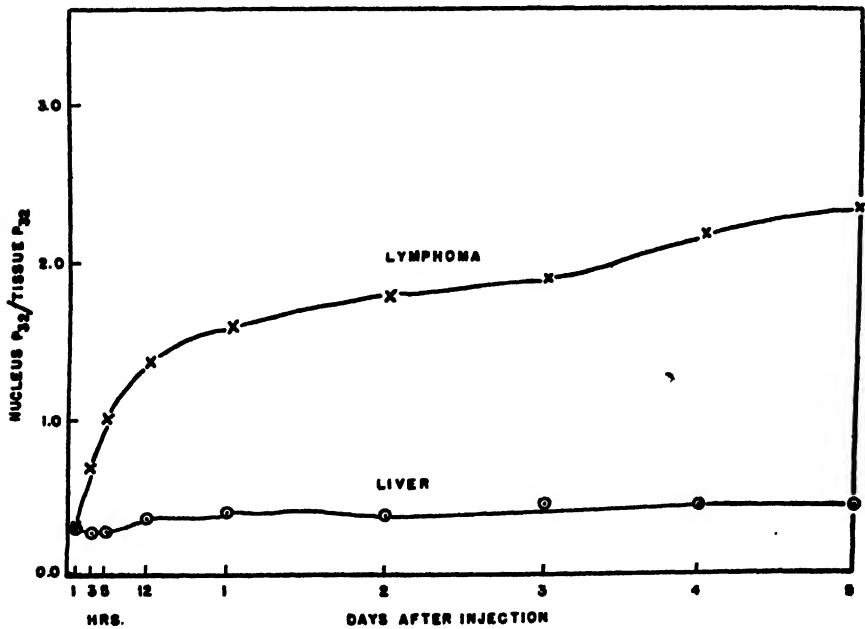
FIG. 2.  $P_{32}$  uptake by nuclei

FIG. 3. Nuclear activity as a fraction of tissue activity

slow decrease. The P<sub>32</sub> curve for liver nuclei (Fig. 2) parallels that of the whole tissue, with a maximum at 3 per cent 1 hour after injection, falling off rapidly to 1 per cent. The uptake by the lymphoma nuclei is strikingly different from that of the whole lymphoma tissue or of the liver nuclei. There is a very rapid rise in nuclear P<sub>32</sub> concentration from 1 per cent at 1 hour after injection to 6.5 per cent at 12 hours. It continues to rise slowly till the 2nd day and then falls off gradually.

When the activity per cubic centimeter of packed nuclei as a fraction of the total activity per gram tissue (Fig. 3) is plotted as a function of time after injection, a striking difference in the behavior of tumor and liver nuclei becomes apparent. The relative concentration of P<sub>32</sub> in the liver nuclei remains practically constant between 1 hour and 5 days after injection, while the concentration in the lymphoma nuclei rises rapidly from 1 to 5 hours after injection and continues to rise as late as 5 days afterward. The P<sub>32</sub> concentration in the lymphoma nuclei is doubled in 2 hours and tripled in 5 hours. Evidently in the liver cell phosphorus not only enters the nucleus rapidly but also leaves it rapidly maintaining a steady state in the interchange of phosphorus between nucleus and cytoplasm. In the lymphoma cell the phosphorus enters the nucleus rapidly and there is no direct evidence from the curves of P<sub>32</sub> uptake of a rapid loss of phosphorus by the nuclei.

#### *Distribution of P<sub>31</sub> and P<sub>32</sub> in Nucleus and Cytoplasm*

Table II shows the phosphorus content of liver and tumor tissues and the corresponding nuclei, determined by the Pregl (10) method. The number of determinations on different samples is given by the columns *n*.

The volume of the packed mass of nuclei in each suspension was obtained by centrifugation in Bauer and Shenck tubes as previously described and corrected to the true volume of nuclei by multiplying by the maximum packing fraction for spheres (0.74). By dividing these values by the number of nuclei per cubic centimeter of suspension the average volume of an individual nucleus was obtained. The average volume of a liver nucleus so obtained was  $3.26 \pm 0.128 \times 10^{-10}$  cm.<sup>3</sup> (*n* = 31) and of a lymphoma nucleus,  $1.673 \pm 0.060 \times 10^{-10}$  cm.<sup>3</sup> (*n* = 30). The densities of the nuclei determined by means of a pycnometer were 1.148 for the liver and 1.146 for the lymphoma nuclei. From these values the P<sub>31</sub>/gm. nuclei was calculated.

The phosphorus content of lymphoma cells was calculated as follows: The diameters of 30 lymphoma cells which had been immersed in 5 per cent citric acid for half an hour were measured with an ocular micrometer. The mean diameter was  $9.75 \pm 0.24 \times 10^{-4}$  cm. from which, assuming a spherical shape for the cell, a volume of  $4.85 \times 10^{-10}$  cm.<sup>3</sup> was obtained. With no phosphorus in the cytoplasm, the P<sub>31</sub>/gm. tissue would be:

$$P_{31}/\text{gm. nuclei} \times \frac{1.67}{4.85} = 2.30 \text{ mg.}$$

The tissue was found to have 2.995 mg. P/gm. and therefore the cytoplasm in 1 gram of tissue must have contained 0.695 mg. P. The  $P_{32}$  in the cytoplasm may be calculated in the same way.

The ratio  $\frac{\text{Nuclear } P_{32}}{\text{Tissue } P_{32}}$  tumor reaches a value of 2.35 by the 5th day after injection of  $P_{32}$ , which is approximately the value (2.24) found for the ratio  $\frac{\text{Nuclear } P_{31}}{\text{Tissue } P_{31}}$ . Thus after a relatively long period of time the distribution of  $P_{32}$  in the cell becomes the same as that of  $P_{31}$  already present.

The complexity of the structure of the liver makes it more difficult to obtain an accurate measurement of the relative volume of nuclei and cytoplasm. However, a rough estimate has been obtained. When the suspension of pulped liver in 5 per cent citric acid is centrifuged and the clumps of cells are removed, the remaining nuclei are of three principal types easily distinguishable, liver, lymph, and endothelial; the latter two being more frequent in the upper layers

TABLE II  
*P<sub>31</sub> Content in Milligrams*

Liver	n	Tumor	n
Tissue, 1 gm. $2.882 \pm 0.069$	26	$2.995 \pm 0.106$	19
Nuclei, 1 cc. $4.53 \pm 0.248$	25	$7.66 \pm 0.234$	18
Nuclei, 1 gm. $3.95 \pm 0.216$		$6.69 \pm 0.204$	

of the centrifuged mass of nuclei. Counts of suspensions of nuclei from normal mouse livers showed the relative frequencies of the three types of nuclei to be 35, 39, and 16 respectively. The mean diameter of the liver nuclei in 5 per cent citric acid was found to be  $11.05 \pm 0.27 \times 10^{-4}$  cm.<sup>3</sup> and in 0.9 per cent NaCl  $12.05 \pm 0.33 \times 10^{-4}$  cm.<sup>3</sup>. Liver cells are approximately parallelopipeds, the lengths of two sides of which were found to be  $26.1 \pm 0.5 \times 10^{-4}$  cm. and  $17.7 \pm 0.5 \times 10^{-4}$  cm. in citric acid and  $26.6 \pm 0.7 \times 10^{-4}$  and  $21.5 \pm 0.6 \times 10^{-4}$  cm. in saline. (Evidently the citric acid produces little or no change in size of either the nucleus or the whole cell.) From these measurements the volumes of the liver cell and nucleus were found to be  $12.1 \times 10^{-9}$  cm.<sup>3</sup> and  $7.26 \times 10^{-10}$  cm.<sup>3</sup> respectively. The endothelial nuclei are approximate prolate spheroids and have a volume of about  $1.45 \times 10^{-10}$  cm.<sup>3</sup>. The volume of the lymph cell has already been given and the endothelial cell assumed to have approximately the same volume. It then becomes apparent from the relative frequencies and the volumes of the different types of cells, that the lymph cells represent only about 4.5 per cent and the endothelial about 2 per cent of the total volume of liver tissue. Therefore as a first approximation we may assume that practically all of the  $P_{32}$  found in the liver tissue is in the liver cells. The distribution of  $P_{31}$  in the cell and the specific activities of nucleus and



cytoplasm may be calculated in the same way as for the lymphoma. The calculation given in Table III is based on the volume of the liver cell nucleus as determined by measurement with the microscope ( $7.26 \times 10^{-10}$  cm.<sup>3</sup>), and not the mean nuclear volume of the suspensions of nuclei from the liver which

TABLE III  
Activities As Per Cent of Injected Dose

Time after injection		P <sub>32</sub> /gm.			P <sub>32</sub> /mg.P <sub>31</sub> /gm.			
		Tissue	Nuclei	Cytoplasm	Tissue	Nuclei	Cytoplasm	Nuclei/cytoplasm
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Lymphoma	1 hr.	3.72	1.48	3.21	1.24	0.22	4.63	0.0475
	3 "	3.90	3.03	2.86	1.30	0.45	4.12	0.109
	5 "	4.16	5.72	2.19	1.39	0.86	3.15	0.273
	12 "	3.70	7.58	1.10	1.23	1.13	1.58	0.715
	1 "	4.11	7.55	1.52	1.37	1.12	2.19	0.511
	2 "	4.06	8.45	1.15	1.36	1.26	1.66	0.760
	3 "	3.68	8.29	1.83	1.23	1.24	1.20	1.03
	4 "	2.80	7.17	0.34	0.94	1.07	0.49	2.18
5 "	2.27	6.45	0.05	0.76	0.97	0.07	13.9	
Liver	1 hr.	9.57	3.53	9.36	3.32	0.90	3.52	0.256
	3 "	6.24	2.08	6.11	2.16	0.53	2.31	0.230
	5 "	5.83	1.96	5.67	2.02	0.50	2.16	0.232
	12 "	3.45	1.47	3.36	1.20	0.37	1.27	0.291
	1 day	3.04	1.45	2.94	1.05	0.37	1.11	0.334
	2 "	1.96	1.11	1.89	0.68	0.28	0.71	0.394
	3 "	2.12	1.14	2.05	0.74	0.29	0.78	0.372
	4 "	2.34	1.21	2.27	0.81	0.31	0.86	0.360
5 "	1.61	0.84	1.56	0.56	0.21	0.59	0.356	

mg. P<sub>31</sub>/gm.

	Tissue	Nuclei	Cytoplasm
Lymphoma.....	2.995	6.69	0.695
Liver.....	2.882	3.95	2.644

were used ( $3.26 \times 10^{-10}$  cm.<sup>3</sup>). Thus with no phosphorus in the cytoplasm the P<sub>31</sub>/gm. liver tissue would be:

$$P_{31}/gm. \times \frac{0.726}{12.05} = 3.95 \times 0.0604 = 0.238 \text{ mg.}$$

The cytoplasm therefore contains 2.64 mg. P<sub>31</sub>/gm. tissue. If the mean volume of the different types of nuclei from the liver is used the P<sub>31</sub>/gm. tissue due to nuclei is 0.108 and the cytoplasm has 2.77 mg. P<sub>31</sub>/gm.

From Table III it is apparent that the concentration of P<sub>32</sub> in the nuclei of the

lymphoma is already about 50 per cent of that in the cytoplasm at 1 hour after injection. 2 hours later the nuclear  $P_{32}$  content has doubled and is greater than that of the cytoplasm. By 5 days after injection practically all the lymphoma  $P_{32}$  is in the nuclei. In the liver the  $P_{32}$  content of the nuclei is about 35 per cent of the cytoplasmic  $P_{32}$  shortly after injection and is 50–60 per cent at 2–5 days after injection. In the lymphoma there is a rapid concentration of  $P_{32}$  in the nuclei accompanied by a depletion of the cytoplasmic  $P_{32}$ . The rate of decrease of cytoplasmic  $P_{32}$  is less than the rate of increase of nuclear  $P_{32}$ . This would be expected if (1) phosphorus moved out of the nucleus into the cytoplasm or if (2) phosphorus enters the cytoplasm more rapidly than it is bound by the nucleus or if both conditions obtain.

### *Specific Activities*

Although the lymphoma nuclei show a rapid accumulation of  $P_{32}$ , the cytoplasm of the lymphoma has a greater specific per cent activity (per cent  $P_{32}$ /mg.  $P_{31}$ ).<sup>1</sup> The lymphoma cytoplasm has a greater s.p.a. than the liver cytoplasm at all the intervals after injection except the last 2 days. If phosphorus enters the cytoplasm more rapidly than it is bound by the nucleus we would expect not only a greater initial concentration of  $P_{32}$  in the cytoplasm, but also a greater dilution of the cytoplasmic  $P_{32}$  by  $P_{31}$  in the later intervals after injection. This expectation is fulfilled since on the 5th day when the specific activity of the lymphoma nuclei is approximately 1, that of the cytoplasm is only 0.07. While in the lymphoma there is a rise in the s.p.a. of the nuclei with a corresponding decrease for the cytoplasm, in the liver both nuclei and cytoplasm decrease in specific activity after the 1st hour. Nevertheless there is a gradual increase in the relative s.p.a. of the liver nuclei by a factor of about 1.4 in 5 days (Table III, column 8) which indicates that the nuclei of the liver are synthesizing relatively non-labile phosphorus compounds even though no cell division takes place. In the lymphoma the concentration of  $P_{32}$  in the nucleus is much more striking, the relative specific activities of nucleus and cytoplasm increasing from 0.048 to 13.9 in 5 days, that is, by a factor of about 290.

### *$P_{32}$ Uptake in Relation to Mitosis*

To determine whether the rapid concentration of  $P_{32}$  in the nuclei is peculiar to the lymphoma, similar experiments were performed with mice of the Swiss strain carrying bilateral sarcoma 180 tumors. The results given in Table IV show that  $P_{32}$  is rapidly accumulated by the sarcoma nuclei in much the same way as was observed for lymphoma nuclei.

<sup>1</sup> Since specific activity has been defined as microcuries of activity per milligram we will use the term specific per cent activity (s.p.a.) as the activity in per cent of the injected dose per mg.  $P_{31}$ .

In order to determine whether the rapid phosphorus accumulation by nuclei is peculiar to tumor cells or is characteristic of mitotic activity of cells, the following experiment was performed. The median and left lateral lobes of the livers of three 150 gm. rats were removed and P<sub>32</sub> as isotonic Na<sub>2</sub>HPO<sub>4</sub> injected by way of the femoral vein 36 hours later. 3 normal rats and 3 rats carrying bilateral carcinoma 256 implants were injected at the same time. 2 days later the livers were cleared of blood by perfusion with saline and the nuclei isolated from the livers and tumors. For the normal liver the ratio of activity per cubic centimeter of nuclei to activity per gram tissue was 0.345, while for regenerating liver and tumor the ratios were 1.02 and 1.08 respectively. When the P<sub>32</sub> was injected 4 days after partial hepatectomy and the nuclei removed 3 days later the ratios for normal and for hepatectomized animals were 0.28 and 0.32. At this time very few nuclei were found in mitosis, while at 36 hours after partial

TABLE IV  
*Sarcoma 180*  
*Activity As Per Cent of Injected Dose*

Time after injection	Activity/gm. tissue		Activity/cc. nuclei		Activity nuclei/ activity tissue	
	Liver	Tumor	Liver	Tumor	Liver	Tumor
<i>hrs.</i>						
3	7.47	1.93	2.84	1.01	0.381	0.527
5	6.24	3.12	5.32	4.20	0.856	1.35
12	6.16	4.94	3.98	5.97	0.645	1.21

hepatectomy 3.7 per cent were in anaphase or metaphase and a much larger per cent in prophase. The rapid accumulation of P<sub>32</sub> by nuclei may therefore be attributed to mitotic activity and not to metabolism peculiar to certain types of cells.

#### *Distribution of P<sub>32</sub> in Nuclei*

The nuclei from tissues of 10 mice were extracted with 10 cc. of 95 per cent alcohol at 55°C. for 1 hour. They were then placed in 10 cc. of a mixture of 2 parts alcohol and 1 of ether and left at 55°C. overnight and then extracted 3 times with 10 cc. of alcohol-ether allowing 1 hour for each extraction. The extract was then evaporated almost to dryness, taken up in water, and shaken 5 times with petroleum ether. The alcohol extract was also evaporated until the alcohol was removed and extracted with petroleum ether. The nuclear residue was then extracted 3 times with 10 cc. 5 per cent trichloroacetic acid at 5°C.

Table V gives the distribution of P<sub>32</sub> in the different fractions as per cent of the activity in the untreated nuclei. There is considerable variation in the

$P_{32}$  found in the three extracts of the liver nuclei. Only about one-half cc. of packed nuclei were used in each experiment, an amount apparently too small for the limits of error of the procedures used. However, the per cent activity in the residues is consistently about 60-70 per cent of the total nuclear activity.

TABLE V  
*Distribution of  $P_{32}$  in Nuclei*  
 *$P_{32}$  as Per Cent of Nuclear  $P_{32}$*

	Time after injection	Water soluble		Lipid	Acid soluble	Residue	Total
Liver	1 hr.		21.4*		6.0	67.2	94.6
	1 day	15.2		6.1	3.3	68.3	92.9
	2 "		20.8*		13.9	61.7	96.4
	3 "	26.1		4.7	0.8	66.0	97.6
	4 "					68.0	
	5 "	2.7		8.6	1.9	66.9	80.1
	7 "	12.6		7.7	7.7	71.6	99.6
Lymphoma	1 day	2.5		0.6	0.6	94.8	98.5
	2 "	1.5		2.3	0.7	95.4	99.9†
	3 "	3.6		0.8	2.5	94.5	101.4
	4 "	2.0		1.4	5.1	91.6	100.1
	5 "	2.1		0.3	3.4	88.1	93.9
	7 "‡	8.4		1.4	26.4	70.4	106.6

\* In these experiments the water-soluble and lipid fractions were assayed together.

†  $P_{32}$  in each fraction is given as per cent of the sum of all fractions.

‡ High  $P_{32}$  in water- and acid-soluble fractions is paralleled by comparably high  $P_{31}$  values for the same nuclei.  $P_{31}$  and  $P_{32}$  determinations on liver nuclei of the same animals show no increase in these fractions. The rise must therefore be associated with mitotic activity. Ionization by beta particles from  $P_{32}$  produces chromosome fragments which are not included in the daughter nuclei after anaphase but form micronuclei which at the onset of the next cell division become disorganized, stain diffusely, and eventually disappear. A tumor with  $8\mu\text{c/gm.}$  will have 30 per cent of its anaphases with chromosome fragments at 24 hrs. after the  $P_{32}$  is injected and with  $17\mu\text{c}$  will have 50 per cent such abnormal anaphases. (11) If the dissolved material (nucleic acid?) becomes incorporated in the new nuclei or adsorbed on their surfaces the rise in the acid- and water-soluble fractions may be accounted for. See Table VI.

Evidently even in the liver nuclei which do not undergo mitosis the greater portion of the  $P_{32}$  taken up is in the nucleoprotein fraction. Furthermore as early as 1 hour after the administration of the  $P_{32}$  the per cent in the nucleoprotein is as great as at several days after injection so that conversion of cytoplasmic phosphorus to nucleoprotein must be quite rapid.

The residues of the tumor nuclei contain 90-95 per cent of the total nuclear activity at all times until the 7th day after injection of the  $P_{32}$ . Since the tumor

is growing rapidly and therefore synthesizing new nuclei, it is not surprising to find practically all of the P<sub>32</sub> in the nucleoprotein fraction. But in a so called resting nucleus, as in the liver, the phosphorus is also being replaced and since it has been demonstrated that the P<sub>32</sub> is not held in the liver nucleus as the phosphate ion, it follows that nucleic acid or portions of it are continually being broken down and reformed from phosphorus compounds furnished by the cytoplasm.

TABLE VI  
*Distribution of P<sub>32</sub> in Nuclei*  
*Mg./Cc. Packed Nuclei*

Nuclei		Water soluble	Acid soluble	Lipid	Residue	Sum of fractions
Liver	3.85	0.27	0.30	0.32	2.26	3.15
	4.50	0.25	0.13	0.20	3.75	4.33
	m	0.26	0.22	0.26	3.01	3.74
Lymphoma	5.68	0.20	Trace	0.11	5.25	5.56
	7.06	0.18	0.28	0.15	5.25	5.86
	m	0.19	0.25*	0.13	5.25	5.71
7 days after injection of P <sub>32</sub>						
Liver	3.59	0.19	0.40	0.28	2.82	3.69
Lymphoma	6.26	0.96	1.30	0.13	4.15	6.54

\* Analysis of a third batch of nuclei showed 0.23 mg. P/cc. packed nuclei. The mean is therefore taken at 0.25. The deductions remain unchanged whether 0.14 or 0.25 is used.

TABLE VII

	Fractions as per cent of Total						S. P. A.		
	P <sub>31</sub>			P <sub>32</sub>			P <sub>32</sub> /P <sub>31</sub>		
	H <sub>2</sub> O and acid	Lipid	Resi- due	H <sub>2</sub> O and acid	Lipid	Resi- due	H <sub>2</sub> O and acid	Lipid	Resi- due
Liver...	12.8	7.0	80.5	18.4	7.5	71.2	1.44	1.07	0.89
Lymphoma.....	7.7	2.3	92.0	4.9	1.1	94.0	0.64	0.48	1.02

Table VI gives the distribution of phosphorus in the nuclear fractions as determined by the Pregl method. Approximately 0.2–0.5 gm. nuclei were used in each of the lots extracted.

In Table VII the P<sub>31</sub> content of the nuclear fractions is given as per cent of the sum of the fractions. The P<sub>32</sub> is given as the mean per cent of the sum of the P<sub>32</sub> fractions from 1 hour to 5 days after administration of the phosphorus. This procedure is not justifiable in the case of the acid-soluble fraction in the lymphoma nuclei which shows a very definite increase with time.

The quantity of nuclei used was too small for an accurate determination of the phosphorus distribution, but is adequate for a rough preliminary estimate. In the liver nuclei the acid-soluble fraction has the greatest specific activity and the nucleoprotein the least. In the lymphoma, as might be expected, the specific activity of the nucleoprotein is about twice as great as that of the other two fractions. If the specific activity of the trichloroacetic acid-soluble fraction is calculated separately from the water-soluble fraction we find that it rises from 0.14 on the 1st day after  $P_{32}$  injection to 1.17 on the 4th day and 5.9 on the 7th day. The water-soluble fraction has a specific activity of 0.57 and shows no comparable increase with time until the 7th day.

### *Rate of P Replacement in Nuclei*

The time required by the nuclei of the lymphoma to bind a quantity of phosphorus equal to that which they initially contained is the time required for the synthesis of an equal quantity of nuclei and should correspond to the time it takes the tumor to double its size. The time for complete replacement of the nuclear  $P_{31}$  may be calculated. Let:-

$U_{P_{32}} = P_{32}$  uptake by nuclei as per cent/gm. of the injected dose.

$C_{P_{31}} = P_{32}$  uptake by cytoplasm as per cent/gm. of the injected dose.

$C_{P_{31}} =$  Total  $P_{31}$  in the cytoplasm in milligrams.

$U_{P_{31}} = P_{31}$  uptake by nuclei in milligrams.

Using the value for  $C_{P_{32}}/C_{P_{31}}$  observed at 1 hour after  $P_{32}$  injection, if all the cytoplasmic P is available to the nucleus:

$$U_{P_{31}} = C_{P_{31}} \times \frac{U_{P_{32}}}{C_{P_{32}}} = \frac{C_{P_{31}}}{C_{P_{32}}} \times U_{P_{32}} = 0.24 \text{ mg. } P_{31}/\text{hr.}$$

Since the nucleus contains 6.7 mg. P/gm. the time for complete replacement is 27 hours. The growth curve of the lymphoma (12) shows that a 14 day old tumor (0.6 gm.) will double its size in about 24 hours. The agreement is surprisingly good for an approximation but may be accidental.

### *Rate of Synthesis of Nucleotides<sup>3</sup>*

From the size of the lymphoma nucleus  $1.67 \times 10^{-10}$  cm.<sup>3</sup> and the 7.66 mg. P/cc. nuclei, a single tumor nucleus is estimated as having  $12.8 \times 10^{-10}$  mg. A lymphoma nucleus will thus contain  $129 \times 10^{-10}$  mg. or  $62.5 \times 10^8$  molecules of tetranucleotide, and if it takes 24 hours for a new nucleus to be formed,  $7 \times 10^4$  molecules of tetranucleotide must be synthesized per second.

### *Effect of X-Ray on $P_{32}$ Uptake*

The decrease in  $P_{32}$  content of liver nuclei shortly after administration of the  $P_{32}$  suggests a movement of phosphorus out of the nucleus as well as into it. A

similar process may occur in the lymphoma but be obscured by the rapid accumulation of  $P_{32}$  due to the synthesis of new nuclear materials. If the outward movement could be interrupted an increase in the  $P_{32}$  concentration in the nuclei is expected. Mice carrying 13 day old lymphomas were given 200 roentgens,  $P_{32}$  injected intravenously one-half hour later, and tissues and nuclei isolated in the usual way at various intervals afterward. The results are given in Table VIII. At 2 hours after the x-ray treatment the  $P_{32}$  concentration in the lymphoma nuclei is 1.6 times that of the controls and then gradually approaches the control value. When the x-ray dose is increased to 260 r. the ratio of  $P_{32}$  concentration in the lymphoma nuclei is increased from 1.7 to 2.1 at 3 hours.

TABLE VIII  
 *$P_{32}$  Uptake Following X-Ray  
207 r.—13 Day Tumors*

Time after irradiation	Tissue per cent injected dose				Nuclei per cent injected dose				Nuclei/tissue			
	Liver	Control	Tumor	Control	Liver	Control	Tumor	Control	Liver	Control	Tumor	Control
<i>hrs.</i>												
2	6.16	7.2	3.54	3.9	1.52	2.0	2.56	1.60	0.246	0.30	0.72	0.46
3	5.75	6.5	3.54	3.9	1.66	1.75	3.66	2.50	0.288	0.28	1.03	0.60
6	5.65	5.8	3.48	3.9	1.71	1.65	5.38	4.9	0.274	0.30	1.558	1.02
12	4.05	4.0	3.82	3.9	1.54	1.40	7.45	6.1	0.381	0.36	1.95	1.36
24	2.92	3.0	3.30	3.9	0.815	1.20	7.19	6.8	0.280	0.40	2.18	1.60
260 r.—15 day tumors												
3	5.15	6.5	3.79	3.9	2.12	1.75	5.17	2.50	0.412	0.28	1.36	0.60

After irradiation with x-rays a considerable proportion of the nuclei becomes pycnotic. To determine whether the increased accumulation of  $P_{32}$  in the nuclei might be a consequence of the pycnotic condition, mice were given 200 r. and the tumors removed and examined microscopically at 1/2, 1, 2, 3, 6, and 8 hours after irradiation. There is a small percentage of pycnotic nuclei in the non-irradiated control tumors. After irradiation there is no noticeable increase in pycnosis the first 2 hours after irradiation, at 3 hours there is a slight increase, which becomes very marked by the 6th and 8th hours. Since the maximum increase in  $P_{32}$  concentration is observed at 2 hours after irradiation this effect cannot be a consequence of pycnosis.

Table IX shows that the  $P_{32}$  concentration of the irradiated tumor tissue remains consistently about the same or slightly less than that of the control tumor. In the irradiated nuclei the concentration is about 60 per cent greater than the control at 2 hours after irradiation, then decreases to a value 10–20 per cent greater than the control at 5–24 hours. The irradiated cytoplasm on

the other hand has 20 per cent less than the control at 2 hours and decreases to 40-60 per cent less than the control at 5-24 hours.

Increase in permeability cannot be invoked as an explanation for the increased nuclear  $P_{32}$  content since the total  $P_{32}$  content of the tissue is not increased. The altered distribution of the  $P_{32}$  may then be attributed either to increased uptake by the nucleus or to decrease in movement from the nucleus to the cytoplasm. Since the irradiated liver nuclei show less than the controls the rate of movement of  $P_{32}$  out of these nuclei cannot be inhibited unless we assume a simultaneous decrease in rate of movement into the nuclei.

Whatever may be the immediate cause of the altered distribution of  $P_{32}$  in the cell it is clear that the x-ray effect is non-existent or small in the liver but pronounced in the tumor. As previously pointed out the difference in normal  $P_{32}$  uptake by nuclei of liver and lymphoma is to be attributed to active

TABLE IX  
*Distribution of  $P_{32}$  in Lymphoma following X-Ray*  
*Per Cent  $P_{32}$ /Gm.*

Time after irradiation	Tissue			Nuclei			Cytoplasm		
	Control	X-ray	X/C	Control	X-ray	X/C	Control	X-ray	X/C
<i>hrs.</i>									
2	3.9	3.5	0.90	1.9	3.0	1.6	3.1	2.5	0.8
3	3.9	3.5	0.90	3.0	4.3	1.5	2.9	2.1	0.7
6	3.9	3.5	0.90	5.8	6.3	1.1	2.2	1.3	0.6
12	3.9	3.8	0.98	7.2	8.8	1.2	1.1	0.8	0.7
24	3.9	3.3	0.85	8.0	8.5	1.1	1.1	0.5	0.4

mitosis in the latter. The concentration of phosphorus in the lymphoma nuclei after irradiation may therefore be associated in some way with mitotic activity. X-rays will inhibit the onset of mitosis, arresting the nuclei in the resting stage. Sometime later than 3 hours after irradiation metaphase and anaphase nuclei begin to reappear. The results therefore suggest a correlation between the increased concentration of phosphorus in the nuclei and the inhibition of mitosis.

#### DISCUSSION

Hahn and Hevesy (13) studied the rate of turnover of nucleic acid in rabbit liver with the aid of  $P_{32}$ . They found that although other organic compounds of the liver were completely renewed in a few days only 1/3 of the nucleic acid phosphorus is replaced after as much as 50 days. They also suggest that the low specific activity of the nucleic acid may be due to a possible slow penetration of inorganic phosphorus into the nucleus. This is not supported by the results of the experiments reported here, which show that the nuclei



contain 4.1 per cent/gm. of the injected  $P_{32}$  at 1 hour after injection and 65 per cent of this is already converted into nucleoprotein. The rate of  $P_{32}$  uptake by the liver nuclei is greater than that of the lymphoma. In the latter there is complete  $P_{31}$  replacement in about 1 day. It follows that in the liver nuclei there is complete replacement in 1 day or less. In these experiments it has been shown that there is little or no exchange between inorganic phosphorus and the nuclei. Hahn and Hevesy have shown that there is no *in vitro* exchange between thymus nucleic acid and inorganic phosphorus. From the results of both experiments we may conclude that phosphorus enters the liver nucleus by replacement of nucleic acid or portions of it. An explanation of the apparent discrepancy in the specific activities of nucleic acid found in the two experiments may lie in the fact that the liver nuclei not only pick up phosphorus rapidly but give it up rapidly as well and since Hahn and Hevesy's conclusion was based on calculations of the nucleic acid  $P_{32}$  found in the liver at 24 hours after it was administered, the maximum rate was not observed.

Tuttle, Erf, and Lawrence studied the phosphorus metabolism of normal and leukemic tissues with the aid of  $P_{32}$ . They found a rapid conversion of the inorganic phosphorus to nucleoprotein by the lymphoma and were of the opinion that this was too rapid to be accounted for by growth alone (14). In these experiments it has been demonstrated that there is good agreement between the rate of  $P_{32}$  uptake by nuclei and the rate of growth of the tumor. Furthermore, when a tissue such as the liver is stimulated to a high rate of mitotic activity there is a corresponding rise in the rate of  $P_{32}$  incorporation into nuclei. However, it is also true that a tissue (liver) showing no mitosis will also convert inorganic  $P_{32}$  to nucleoprotein and also, a rapidly growing tissue (lymphoma) has cytoplasm with a high specific activity. Apparently synthetic processes and glycolytic processes in the cell are interrelated and cannot be treated as though independent. Such synthesis is evident in the replacement of nucleoprotein even in nuclei which do not undergo mitosis.

#### SUMMARY

1. A method for isolating nuclei in quantity from mammalian tissues is described.
2. The rate of uptake of radioactive phosphorus by nuclei is found to be quite rapid. The phosphorus was shown not to be taken up by exchange.
3. Nuclei of tumors accumulate more radioactive phosphorus than normal liver nuclei. This was shown to be due to mitotic activity and not a form of metabolism peculiar to tumor cells.
4. The specific activities of nuclei and cytoplasm are compared.
5. 60 to 70 per cent of the nuclear radioactive phosphorus is present as nucleoprotein from 1 hour to 5 days after it is administered. In the lymphoma

nuclei 90–95 per cent of the phosphorus is in the nucleoprotein fraction from 1–5 days after it is administered.

6. The specific activities of the nucleoprotein, lipid, and acid-soluble fractions of liver and tumor nuclei are compared.

7. From the rate of  $P_{32}$  uptake by nuclei it is calculated that a new lymphoma nucleus is synthesized on the average once every 27 hours. This is in agreement with the observed rate of growth of the tumor.

8. In the lymphoma nucleus it is calculated that  $7 \times 10^4$  molecules of tetranucleotide are synthesized per second.

9. Irradiation with 200 r. x-rays alters the distribution of  $P_{32}$  in the lymphoma cell, markedly increasing the concentration in the nucleus shortly after irradiation. The  $P_{32}$  concentration in the cytoplasm decreases with time after irradiation. It is suggested that the altered distribution is correlated with the inhibition of mitosis produced by the x-rays.

10. Continual synthesis of nucleoprotein takes place even in nuclei of cells which do not undergo mitosis.

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# THEORY AND MEASUREMENT OF VISUAL MECHANISMS

## VII. THE FLICKER RESPONSE FUNCTION OUTSIDE THE FOVEA

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### I

Our chief interest in obtaining flicker response contours for excitation of an extrafoveal region has been to modify differentially the parameters of the "rod" and "cone" response curves, and thus to test the general notions already employed for the analytical separation and interpretation of these functions. This involves not merely a test of the respective properties of the two parts of the duplex curve, but also of the interrelations between these components. Such a test can be made in several ways. In one of these, by using an image of the same areal extent (1) centered at the fovea and (2) placed outside it, one necessarily involves changes in the numbers and proportions of cones and rods, as well as changes in the basic excitability *per* (small) unit area and in the area as a whole. The experiment here described was designed to exhibit the resulting differences in the flicker response function, for different wave-length compositions and as a function of the light-time fraction in the flash cycle. These differences are then to be considered in terms of the theory of the nature of the curves. A different manner of carrying out tests of this general import will be considered in a following paper.

To obtain data for the examination of this matter which should be reasonably homogeneous, one observer was used, employing uniocular measurements (W. J. C., left eye) with white, red, and blue lights of the kind already used for the same retinal area (square, *ca.*  $6.13^\circ$  on a side) with foveal fixation, but now fixated with the center of the area  $8.37^\circ$  on the temporal side of the fovea, and  $t_L = 0.10$  and  $0.90$ . In this way it was possible to secure the desired information without too great a time spread. By suitable check measurements it was verified that no appreciable changes in excitability occurred, within the series of tests or in respect to the homologous measurements with foveal fixation already considered.

The technique of the measurements has been described in some detail (Crozier and Wolf, 1940-41 *a, b*), and the description of the instrumentation (Crozier and Holway, 1938-39 *a*) and procedure will not be given again here. We shall

present the data as compactly as possible, without much theoretical comment, before discussing the deductions to be drawn.

TABLE I

Data for flicker response contours with *white*, W. J. C., monocular (left), 6.3° square centered 8.37° on temporal side of fovea,  $t_L = 0.10, 0.50$ , and 0.90; each entry is the mean of 10 observations;  $I_m$  and P.E.<sub>1f</sub> in *millilamberts*. See Fig. 1.

F  per sec.	$t_L = 0.10$		0.50		0.90	
	log $I_m$	log P.E. <sub>1f</sub>	log $I_m$	log P.E. <sub>1f</sub>	log $I_m$	log P.E. <sub>1f</sub>
2	5.1596	7.5595	5.8616	6.4857	4.4686	6.9235
4	5.3032	7.7701	4.0294	6.5387	4.8726	6.9411
6	5.4763	7.7281	4.1821	6.4865	4.8533	5.3278
8	5.6358	7.9669	4.3150	6.6625	5.0362	5.4631
10	5.7798	6.1906	4.4856	5.0734	5.2030	5.5974
12	5.9348	6.2497	4.6838	5.1519	5.3709	5.6220
14	4.0927	6.5001	4.9302	5.2799	5.5459	5.9528
16	4.3716	6.7843	5.1652	5.6676	5.8090	4.1773
18	4.6821	5.0309	5.4884	5.7830	5.1775	4.3731
19	4.9515	5.2226	5.9179	4.2446	5.5321	4.9132
20	5.5866	4.0672	5.5646	4.8336	5.4118	5.8168
	5.5769	4.0218	5.5895	5.2355	5.3773	5.9123
21			5.7807	5.1543		
22	5.2542	4.5943	5.9294	5.3384	5.7483	5.0447
25	5.3791	4.9142	5.2164	5.7302	5.9556	5.4025
30	5.7372	5.0337	5.5112	5.9822	6.2055	5.6592
35	5.0923	5.4370	5.8644	5.5587	6.5377	5.8508
40	5.5135	5.9188	6.2730	5.8669	6.9607	5.2386
			6.6374	5.6558		
45	6.1501	5.5813	6.8941	5.3195	5.5536	5.7694
48			5.3911	5.8191	5.0962	6.4399
50	5.1062	5.4482	5.7973	6.4452	5.7205	5.0615
	5.0675	5.4890	5.8396	6.3165		
51	5.6218	6.0895	5.4568	6.9644	5.8010	5.0262
	5.7429	6.2821				
52	5.6170	5.0581				
	5.2799	6.8879				

## II

Tables I, II, and III contain the data for *white* at  $t_L = 0.10, 0.50$ , and 0.90, and for *blue* and *red* respectively with  $t_L = 0.10$  and 0.90, for the 6.13° square centered 8.37° on the temporal side of the left fovea. A small red dot suitably located and of suitable intensity provided a foveal fixation point. The square image thus located did not overlap the image of the same size when centered at the fovea. In its position for the present observations the field included the

initial flattish part of the curve of "intrinsic retinal excitability" for this observer, as determined by the use of a quite small test area briefly exposed (Crozier and Holway, 1938-39*b*); when *foveally* centered it included the region of markedly least primary excitability. The *red* light was secured by using Wratten Filter No. 70, the *blue* with No. 47, as in the preceding experiments

TABLE II

*As in Table I, but for Blue at  $t_L = 0.10, 0.90$ ; See Text, and Fig. 9*

<i>F</i> per sec.	$t_L = 0.10$		0.90	
	$\log I_m$	$\log P.E._{1I}$	$\log I_m$	$\log P.E._{1I}$
2			$\bar{7}.3251$	$\bar{7}.1368$
4			$\bar{7}.4968$	$\bar{9}.7693$
6			$\bar{7}.9008$	$\bar{8}.3597$
8			$\bar{6}.2498$	$\bar{8}.7383$
10	$\bar{7}.5037$	$\bar{9}.9117$	$\bar{6}.4504$	$\bar{8}.8495$
12	$\bar{7}.7343$	$\bar{8}.3133$	$\bar{6}.8598$	$\bar{7}.4266$
14	$\bar{6}.1110$	$\bar{8}.6641$	$\bar{5}.2873$	$\bar{7}.6225$
16	$\bar{6}.3707$	$\bar{8}.6936$	$\bar{5}.5998$	$\bar{7}.9959$
18	$\bar{6}.6867$	$\bar{8}.9382$	$\bar{5}.8430$	$\bar{6}.2127$
19	$\bar{5}.3315$	$\bar{8}.9047$	$\bar{4}.4168$	$\bar{6}.7143$
20	$\bar{5}.7811$	$\bar{6}.1931$	$\bar{4}.8688$	$\bar{5}.2423$
	$\bar{5}.7268$	$\bar{6}.1170$	$\bar{4}.8374$	$\bar{5}.2519$
25	$\bar{4}.4925$	$\bar{6}.9613$	$\bar{3}.4550$	$\bar{5}.8431$
30	$\bar{4}.7488$	$\bar{6}.9968$	$\bar{3}.7685$	$\bar{4}.1311$
35	$\bar{3}.1204$	$\bar{5}.6567$	$\bar{2}.1808$	$\bar{4}.5364$
40	$\bar{3}.5448$	$\bar{4}.0975$	$\bar{2}.6662$	$\bar{3}.0945$
45	$\bar{2}.1192$	$\bar{4}.5759$	$\bar{1}.2991$	$\bar{3}.8428$
48			$\bar{1}.6453$	$\bar{2}.0952$
50	$\bar{2}.7469$	$\bar{3}.2094$	$\bar{1}.9514$	$\bar{2}.2459$
52			$0.5305$	$\bar{2}.8740$
53	$\bar{1}.2842$	$\bar{3}.8026$		
54			$1.3160$	$\bar{1}.6953$
55	$\bar{1}.8020$	$\bar{2}.4312$	$2.2186$	$0.4976$
58	$1.2827$	$0.2963$		
	$1.4349$	$1.6105$		

(Crozier and Wolf, 1941-42*a*). The mean intensities given in the tables, and their P.E.<sub>1</sub> values, are in log millilambert units, as obtained from photometric matches with white (below the color level).

The general properties of the  $F$ -log  $I_m$  contours for the extra-foveal situation are similar to those established under conditions of foveal fixation (Crozier and Wolf, 1940-41*b*, 1941-42*a*). The "cone"  $F_{\max}$  is lower for the extra-foveal location, the "rod"  $F_{\max}$  higher. There are a number of quantitative differences in detail. The extent to which these differences can be brought into a

consistent picture of the meaning of the  $F$ -log  $I_m$  contour will be considered in the succeeding sections.

Fig. 1 is a plot of the measurements with *white* at the three light-time fractions. It also contains the extrapolations of the "cone" probability integrals, the "rod" difference curves, and certain information about the subjective character of the end-points. Fig. 2 exhibits the "cone" data on a probability grid. The three slopes here (and consequently  $\sigma'_{\log I}$ ) are the same. The

TABLE III  
As in Tables I and II, but for Red; See Text, and Fig. 10

$F$  per sec.	$t_L = 0.10$		0.90	
	log $I_m$	log P.E. <sub>11</sub>	log $I_m$	log P.E. <sub>11</sub>
2	5.5203	7.8699	4.6020	5.0275
4	5.7061	6.2467	4.7771	5.2129
6	4.0653	6.5448	3.1661	5.5850
8	4.4467	6.7630	3.5668	5.9410
10	4.7827	5.0589	3.8939	4.3626
12	3.0621	5.4702	2.2316	4.5992
14	3.3065	5.6796	2.4203	4.7719
16	3.5813	5.9914	2.7074	3.0515
18	3.8590	4.2270	2.9648	3.3391
20	2.0886	4.3645	1.2148	3.7706
25	2.4174	4.8357	1.5600	3.8851
30	2.6658	4.9158	1.8154	2.1599
35	1.0142	3.4323	0.1438	2.5210
40	1.3901	3.7272	0.5299	2.8889
45	1.9841	2.4205	1.0956	1.6918
48			1.3704	1.7181
50	0.5677	1.0184	1.7079	1.9552
51			1.9316	0.3439
52	0.9962	1.2723		
53	1.7661	0.0331		

values of the "cone"  $F_{\max.}$  are again in rectilinear relation to the light-time fraction (Fig. 3), as are also the magnitudes of the abscissa of inflection  $\tau'$  (Fig. 4).

For the same area of test patch the total number of available primary retinal cone units must be presumed less with the extra-foveal location than for the foveally centered location; but without foveal regard the number concerned in recognition of the end-point could well be greater. It has been pointed out (Crozier and Wolf, 1941-42*a, b*) that there is to be expected, for an increase in the number of these units, a decrease of  $\sigma'_{\log I}$  (*cf.* Crozier, 1940, 1941). The values of  $F_{\max.}$  are a function of the frequencies of effective con-

tributions from these units, as well as of their number. For the foveal and extra-foveal locations we find that the slope in Fig. 2 is actually the greater, and  $\sigma'_{\log I}$  consequently smaller, as expected. The "rod" slope (rising; Fig. 5), here presumably representing the participation of a larger population of rods, might be expected to be less, and is actually about identical or insignificantly greater, for the foveal and extra-foveal locations (*cf.* data in: Crozier and Wolf, 1940-41 *b*). (For the "rod" curve the *apparent*  $F_{\max}$  is the result of

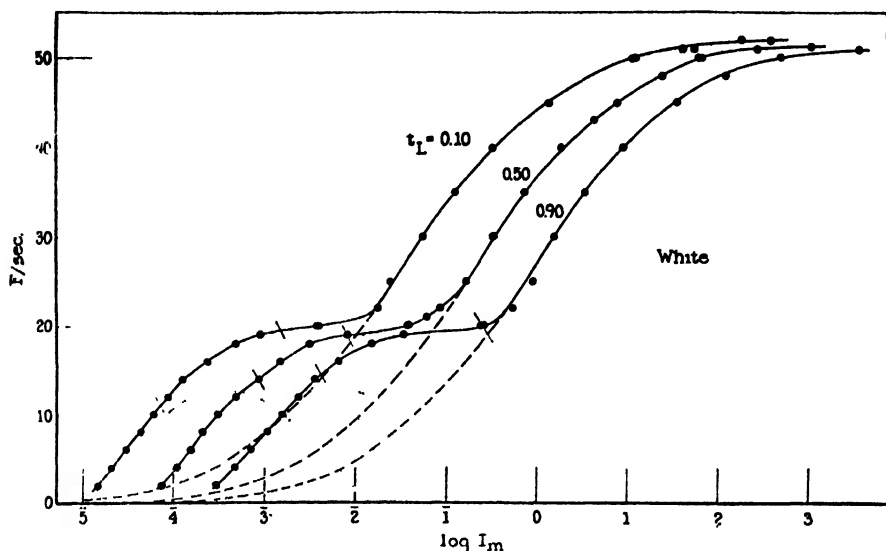


FIG. 1. Data in Table I. Flicker response contours for extra-foveal square, with  $t_L = 0.10, 0.50, 0.90$ ; white light; on each curve the lower mark indicates the upper intensity limit of smooth blue-gray fields; the upper mark indicates transition to a smooth field with "foveal" end-point; between these marks the fields are "speckled." For the "cone" portions of the contours the lines are probability integrals as adjusted in Fig. 2; these are extrapolated toward  $F = 0$ , and the dotted difference-curves ("rod") are also given.

inhibitive "cone" competition; Crozier and Wolf, 1940-41 *a, b*, 1941-42 *a*.) Incidentally, it must be pointed out that in view of the extrapolations (as in Fig. 1, etc.) and difference-taking with curves of different shapes involved in getting at the intrinsic "rod" curves, this kind of quantitative agreement, not expected, is a remarkable fact. It definitely strengthens the view that the procedure used is valid. It may be suggested that, so far as white is concerned, the number of effective "rod" units may well be about the same for the two areas studied.

As a test of the general proposition concerning the effect of a change in the number of "cone" units, we note that in the present series  $F_{\max}$  is much less



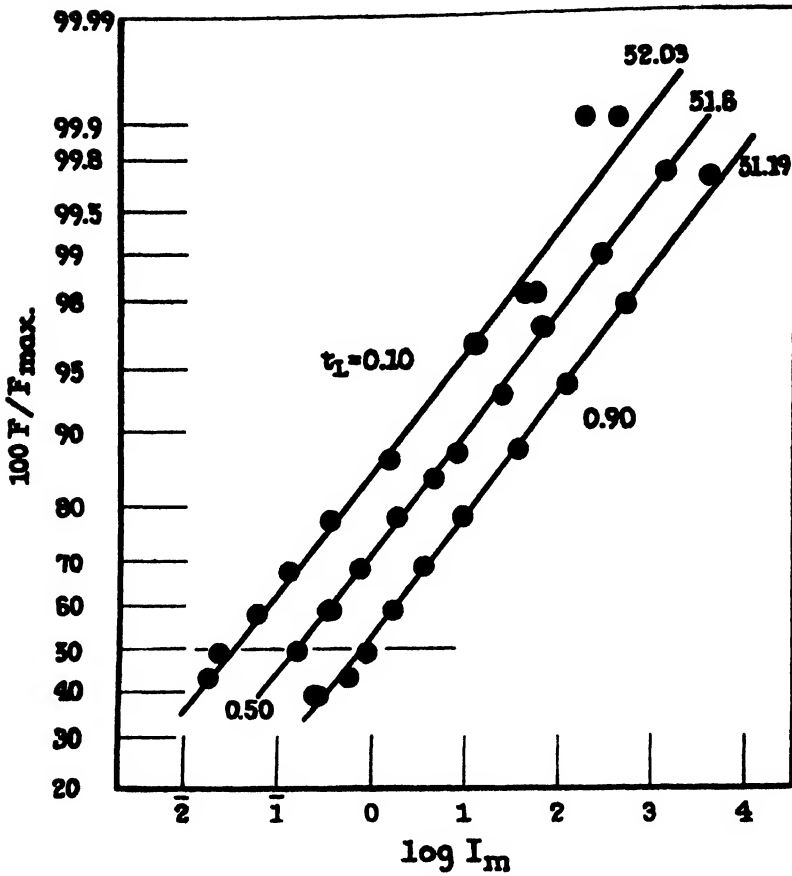


FIG. 2. Measurements in the upper ("cone") parts of the contours in Fig. 1, shown on a probability grid. The number on each graph is the asymptotic value of  $F_{max}$ .

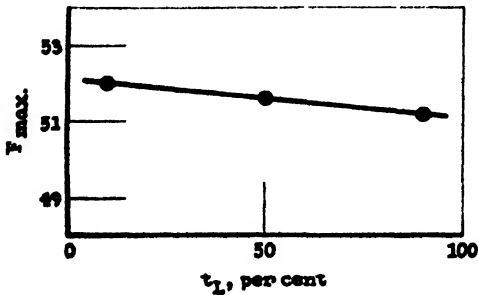


FIG. 3. The rectilinear relation between  $F_{max}$ . (Fig. 2) and the light-time fraction  $t_L$ .

responsive to change of  $t_L$  (Fig. 3; *cf.* Crozier and Wolf, 1940-41 *b*, 1941-42 *a*), although  $\tau'$  is more responsive.

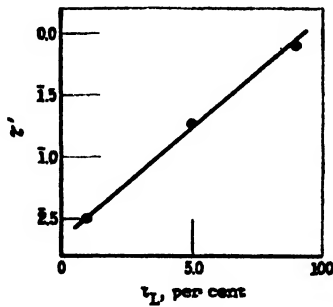


FIG. 4. The rectilinear relationship between the abscissa of infection  $\tau'$  of the "cone" probability curve (Fig. 2) and the light-time percentage.

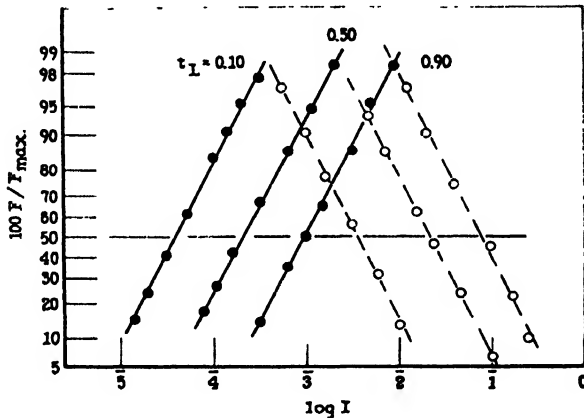


FIG. 5. The rising and descending branches of the difference curves in Fig. 1, shown on a probability grid. Coordinates of points on the dotted curves of Fig. 1 are shown as plotted. The value taken for  $F_{max.}$  is the same for all three curves, namely  $F_{max.} = 12.8/sec.$

The variation data (Table I; Fig. 6) exhibit no unusual features (*cf.* Crozier and Wolf, 1940-41 *a*, *b*). The scatter of P.E.<sub>11</sub> for  $t_L = 0.10$  is smaller, relatively, despite the extra-foveal fixation, than might have been expected. The scatter coefficients ( $\sigma_{P.E.1}$ ) are low, and their relation to  $F_{max.}$  does not differ significantly from those expected by extrapolation (Fig. 7) from the relation established for the foveally centered tests. The proportionality constants for

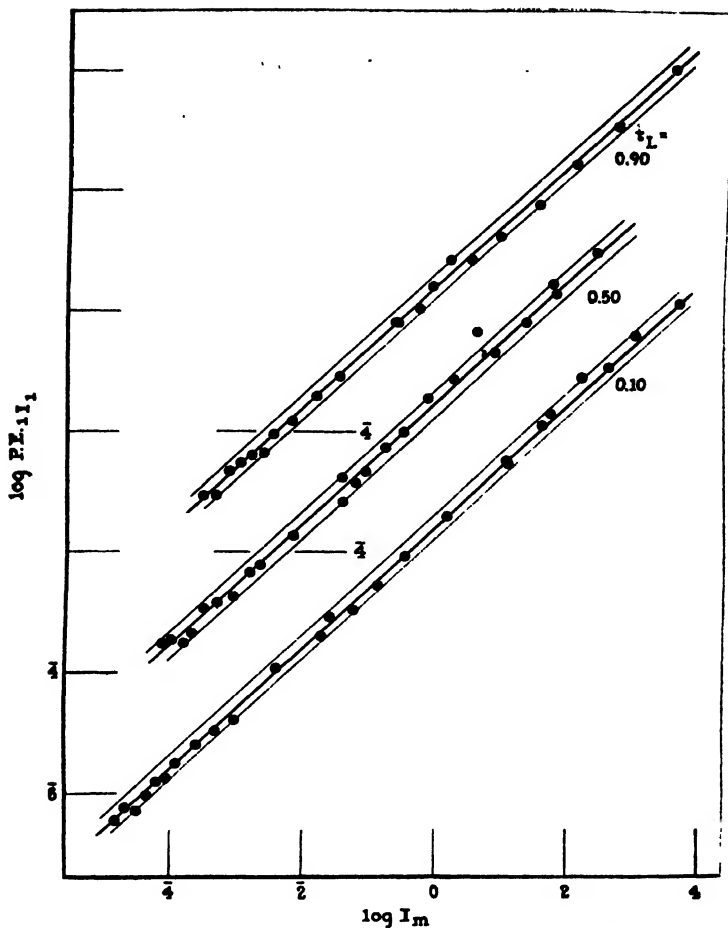


FIG. 6. The relation between  $\log I_m$  and  $\log P.E._{II}$  for the *white* data of Table I and Fig. 1. See text.

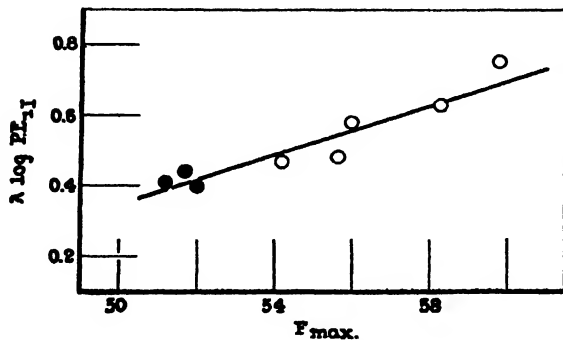


FIG. 7. The relation between the scatter coefficients for  $P.E._{II}$  and  $F_{max.}$  The open circles are from previous measurements with the test square foveally centered, the solid dots for the present measurements (Fig. 6) with the extra-foveal square.

$P.E._{1f} = k I_m$  are in excellent agreement with those already obtained for the foveally centered patch.

As illustrating the complex character of the relationships appearing in a multivariate system of this sort, it is useful to compare the  $F - \log I_m$  curves with the same test area in the two locations used. The essential points appear for any level of  $t_L$ ; we take for illustration (Fig. 8)  $t_L = 0.10$ . It is obvious

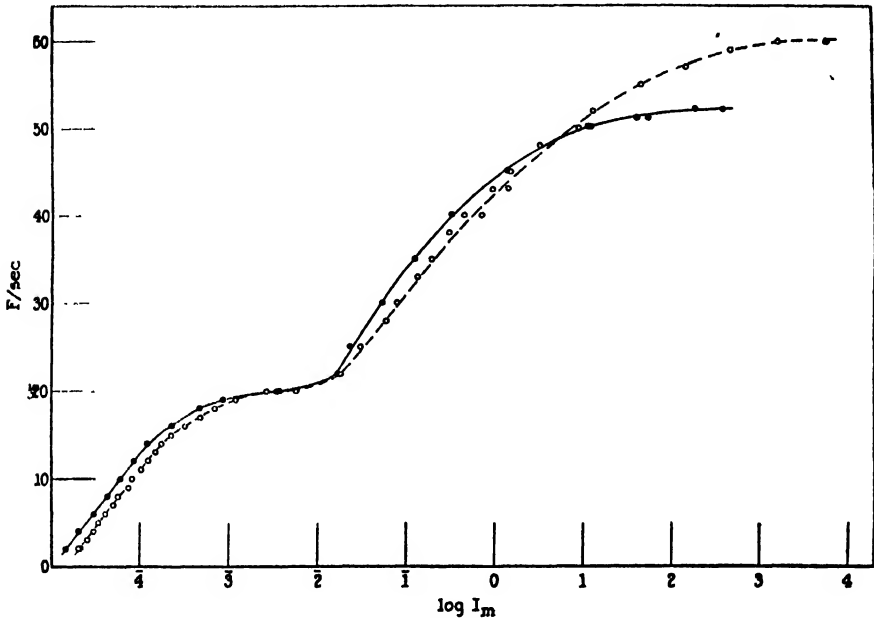


FIG. 8. Comparison of flicker response contours,  $t_L = 0.10$ , for the same square test area centered at the fovea and on the temporal side of the fovea; *white* light, W. J. C., monocular (left eye); open circlets, test square centered at the fovea, solid dots, centered  $8.37^\circ$  on temporal side of the fovea.

that any real comparisons must depend upon the properties of the function as a whole.

### III

The curves for blue and red corresponding to Fig. 1 for white are given in Figs. 9 and 10 (Tables II and III). As with the *white*, the  $F_{max}$  for both *blue* and *red* is less with the foveally centered square; in each case the value of  $F_{max}$  is greater than for *white*. The values of  $\tau'$ , for each  $t_L$ , are well below those for the *white*. The variation indices follow the rules already discussed, and the mean ratio  $k = P.E._1/I_m$  has the same value (Fig. 11).

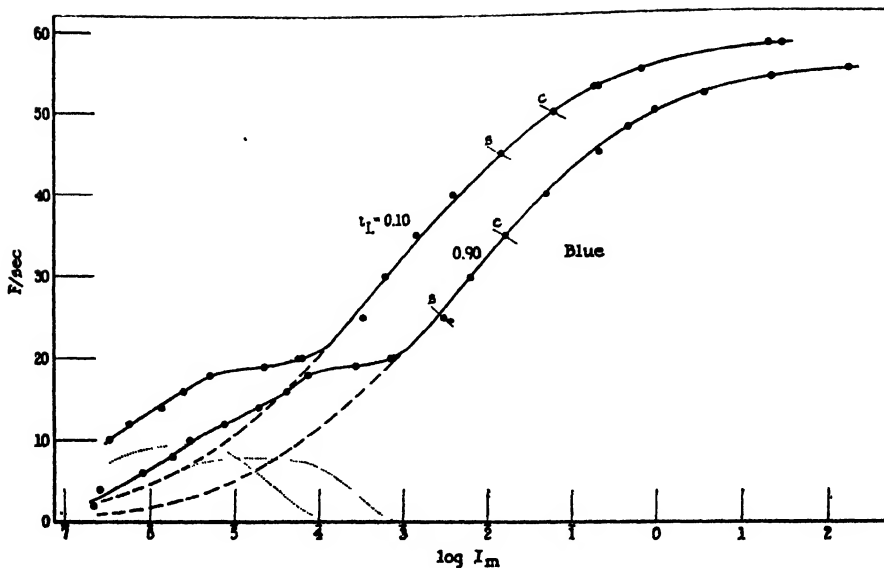


FIG. 9. Flicker response contours for *blue*, extra-foveal test square, with  $t_L = 0.10$  and  $0.90$ . Data in Table II. Above the marks *c*, the end-point fields are colored; above *s*, "smooth."

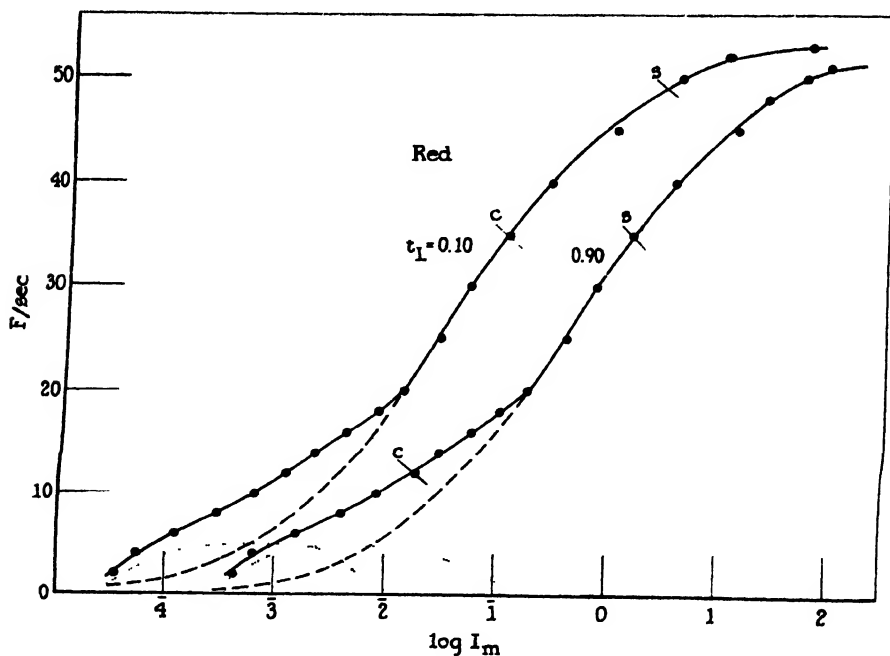


FIG. 10. As in Fig. 9, but for *red*. Data in Table III.

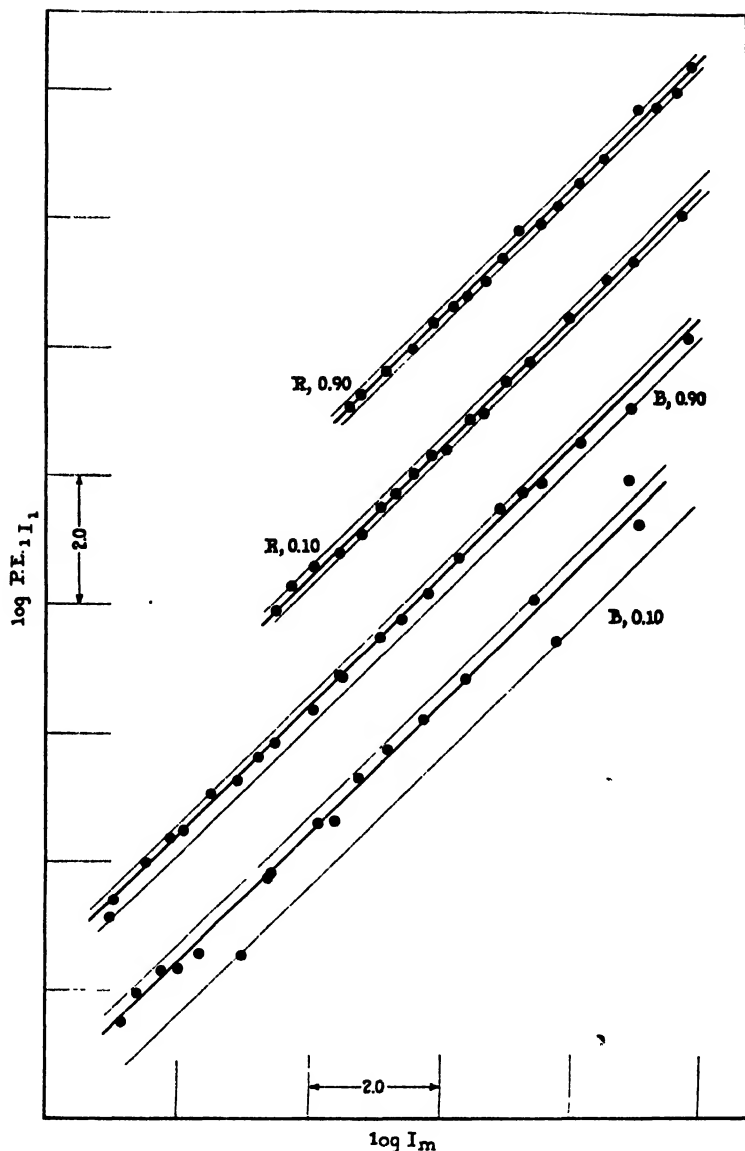


FIG. 11. Variation data for red and blue, Tables II and III. See text.

The probability integrals adjusted to the red and blue data ("cones") show that (Fig. 12), as for the foveally centered case,  $\sigma'_{\log I}$  for the blue is greater than for the red. The red  $\sigma'_{\log I}$  is, like that for white, less than for the square foveally centered; the blue  $\sigma'_{\log I}$ , however, is a very little higher in the present

series, —although the difference is not very significant. Some further information is given by the fact that while in the foveally centered series the *blue* and *red*  $F_{\max}$  are about equally affected (the *blue* a little more) by change of  $t_L$

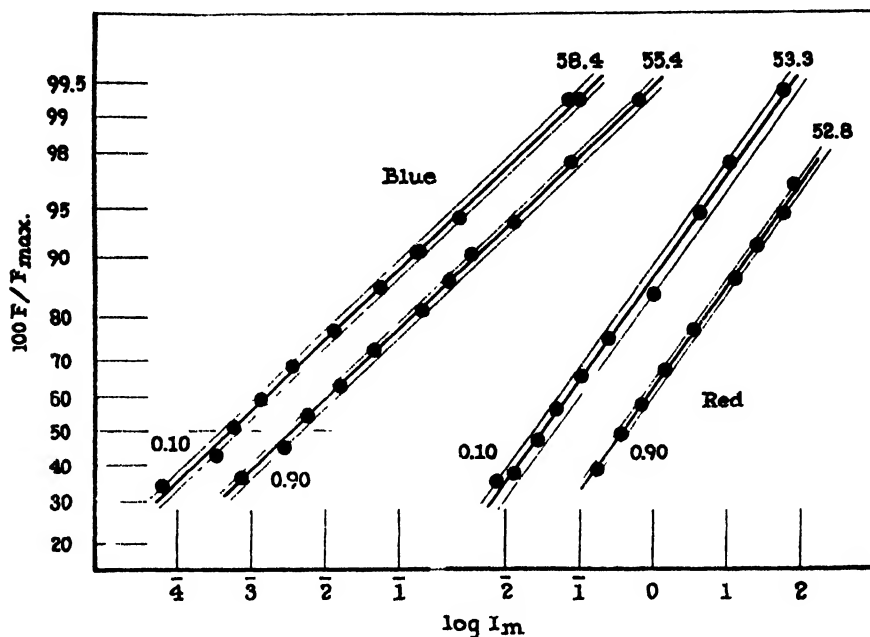


FIG. 12. The upper ("cone") branches of the data in Figs. 9 and 10 put on a probability grid. The associated values of  $F_{\max}$  are indicated.

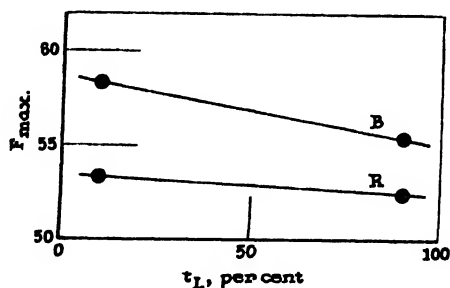


FIG. 13. The relation between  $F_{\max}$  and the light-time fraction, for the *red* and the *blue*.

(Crozier and Wolf, 1941-42*a*), in the present case the effect on the *red* values is proportionately much less (Fig. 13) and less than for the foveally centered test patch, whereas with the *blue* it is slightly more. This is correlated with the fact that the displacement of the test patch to the temporal side of the fovea

reduces the *red*  $F_{\max}$ . more than it does the *blue*, and with the further fact that the effect of change in  $t_L$  upon  $\tau'$  (Fig. 14) is no greater for the *red* and is less for the *blue* than in the centered field.

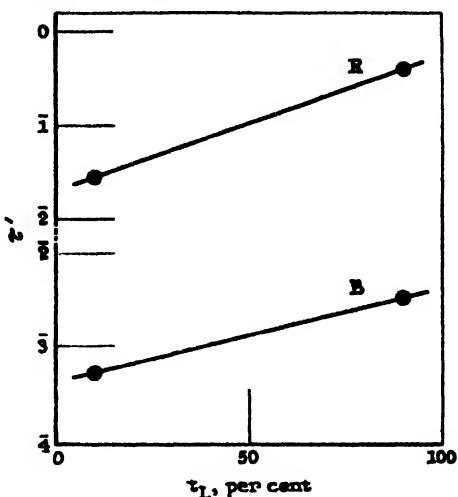


FIG. 14. The relation of "cone"  $\tau'$  to the light-time percentage, for the *red* and the *blue*.

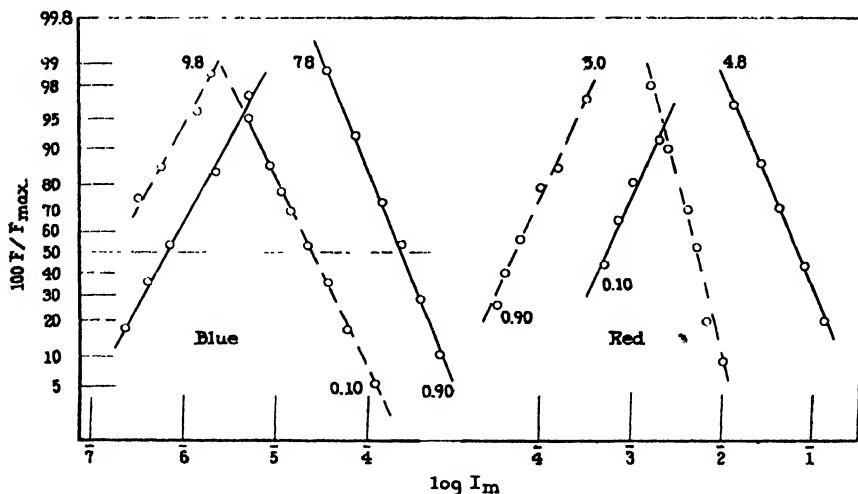


FIG. 15. The "rod" difference-curves of Figs. 9 and 10, for *blue* and *red* respectively, shown on a probability grid.

The dissected-out "rod" curves (Figs. 9 and 10) have been transferred to a probability grid in Fig. 15. The separation of the "rod" and "cone" populations of effects is here great enough so that in each case the apparent "rod"



$F_{\max.}$  declines as  $t_L$  is made greater (*cf.* discussion in: Crozier and Wolf, 1940-41 *b*). The rising "rod"  $\sigma'_{\log I}$  (independent of  $t_L$ ) is *greater* for the *red*, and *less* for the *blue*, than in the foveally centered series. In terms of the view that one expression of the neural integration of "rod" effects is the increasing value of  $\sigma'$  as the number of units involved is made greater, whereas for the "cone" units the reverse is observed (Crozier, 1940; 1941), this could be taken to mean that in moving out from the fovea we have a larger number of red-excitable "rod" units and a smaller number of blue-excitable "rod" units.

We refrain from discussing the question of rod wave-length excitation in detail, because it is obvious that one would require determinations with a series of wave-length compositions and a range of test areas in several different locations. Nevertheless it must be pointed out that the rapidity of the drop of the (small test area) "absolute" threshold for blue, in moving out from the fovea, is considerable,—whereas that for red drops very much more slowly (or, under certain conditions, rises—*cf.* Crozier, Holway and Wolf, 1941-42). This can be made consistent with the indications herein obtained from the properties of the dissected-out "rod" curves, if we remember that the apparent "rod" curve in flicker recognition is the result of a competition with "cone" effects; the proof of this can be obtained by the study of divided fields (Crozier and Wolf, 1941-42 *b*). We note that, in contrast to the situation with the foveally centered patch (Crozier and Wolf, 1941-42 *a*) the  $\log I$  separation of the "rod" and "cone" branches is more extensive for the *red* and rather less for the *blue*.

#### IV

Certain questions raised in our preceding account of the  $F - \log I_m$  contours with the foveally centered test area must be briefly considered in the light of the present measurements, although they cannot as yet be adequately resolved. It was pointed out (Crozier and Wolf, 1941-42 *a*) that a comparison can be made of the energies required to activate one-half the potentially excitable elements ( $dF/d \log I$ ). This was made by estimating the values of  $t_L$  which for various colors and white would bring the ("cone") curves to the same  $F_{\max.}$ , and also the corresponding values of  $\tau'$ . In this respect the mean energy flux for the *white* lay between that for the *red* and the *blue*. In terms of the properties of  $\sigma'_{\log I}$ , also, *white* was intermediate between *blue* and *red*, and thus might be taken to involve numbers of units in the order  $B > W > R$ . The same is true in the present series, the *blue*  $\sigma'_{\log I}$  having increased (a little) in the extra-foveal series and the *red*  $\sigma'_{\log I}$  having decreased.

While it is certainly too early to discuss the possible bearing of these considerations upon the nature of "white," for example, it can be pointed out that if white is taken to be a competitive synthesis of blue, red, *etc.*, then the kind of result here obtained can be rationalized: the intermediate value of the white

$\sigma'_{\log I}$ , the smaller value of its  $F_{\max}$ , under given conditions, and the intermediate value of the mean energy flux for half-activation of the elements of effect potentially excitable. This conception can be more profitably exploited in other connections. Before it can be done efficiently it is desirable to show that the methods used for the analysis of the excitation contours are such as possess generality. There are two distinct aspects to such a demonstration. The nature of the micro-heterogeneous neural mechanism involved in the visual excitation-response phenomena requires that the methods for its analysis have to be non-specific; that is, they must be quantitatively applicable to different kinds of animals. This first requirement is obviously met, with reference to the types of contours obtained for a quite varied assemblage of animals and with reference to the dependence of these contours upon such variables as area excited, temperature, and light-time fraction; we can add to this the essential conformity in the effects of wave-length composition of light (unpublished).

It is equally necessary to show that there prevails the same kind of inner consistency of the analysis when, as can be done with man, different regions of the retina, with known different intrinsic properties, are examined in a single individual. A sign of this consistency is found in the rational relations of the parameters of the complex contour to variables such as those used in the present test. The system of relationships of which this test gives a glimpse is the significant thing, rather than the particular relations between any two variables under conditions otherwise fixed. It has been emphasized that a basic feature of the analysis demands the separation of the properties determined by the *number* of units of a given class potentially excitable, from those properties due to the *frequencies* with which these units contribute to the total effect measured. A procedure for illustrating this separation, and its experimentally measured consequences, is considered in some detail in a following paper (Crozier and Wolf, 1941-42*b*). The meaning of its results can then be utilized for further studies of excitation as a function of wave-length.

## V

### SUMMARY

The several parameters of the flicker response contour ( $F - \log I$ ) are considered as a function of wave-length composition (white, blue, and red) and light-time fraction, for an extra-foveal region (monocular, temporal retina). These data are compared with those secured for the same image area centrally fixated at the fovea. The systematic changes in the parameters are shown to be in rational relation to other relevant excitability data. Since for two retinal regions the primary contours are quite different, the systematic nature of the behavior of the parameters in the two cases is a real test of the power of the analysis proposed. Theoretical interpretation is required to

deal with the properties of sets of performance contours under systematically varied conditions, and cannot rely simply on the comparison of (for example) two contours under the same arbitrary conditions at two retinal locations. In particular it is emphasized that a qualitative separation must be made of the two factors of (a) number of units and (b) the frequencies of their actions, before the wave-length problem can be dealt with effectively.

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# PHOTOXIDATION PROCESSES IN PLANTS

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## INTRODUCTION

Many observations indicate that the uptake of oxygen and the evolution of  $\text{CO}_2$  by plants and plant tissue can be enhanced by irradiation with visible light. Some observers believe that a direct action of the light on the respiratory system is responsible for this effect, which they therefore call "light respiration;" others assume that the increased  $\text{O}_2$  uptake is caused by photooxidation processes not different in principle from analogous effects in non-living matter. Taking in account the results published in the literature and the ones described in this paper we conclude that the second assumption is correct. The ultimate cause of the extra oxygen absorption after or during irradiation consists of photooxidation processes sensitized mainly by chlorophyll, but these processes are by no means simple ones. For instance, the products formed by photooxidation may interfere as substrate or as inhibitor with the normal plant metabolism. We mention as a simple example the fact that by these photooxidation processes the substrate of normal respiration can be chemically changed in such a way that during the subsequent dark period the rate of respiration is temporarily greater than its normal value. According to our opinion the introduction of the term light respiration for such an effect is unnecessary, just as it is for the well known influence which photosynthesis has on respiration in starved plants; by producing carbohydrates photosynthesis restores the absent substrate of respiration and so the rate of respiration measured during the subsequent dark period is increased. We cannot completely preclude the possibility that a genuine light respiration *i.e.*, an influence of light on the system of enzymes involved in respiration, does exist, but observations made with plants by others and by ourselves so far give no clear indication of such an effect. Attempts to find it should be made in gaseous atmospheres containing only about 1 to 2 per cent of oxygen as that oxygen concentration is great enough to saturate respiration yet not large enough to cause significant disturbances by photooxidation processes.

## EXPERIMENTAL PROCEDURE AND RESULTS

The main methods of observing photooxidation processes in normal plant tissue belong to two different groups. The first is an indirect one based on early observa-

tions made by Warburg (1). Photosynthesis at different light intensities is measured as a function of the oxygen content in the gaseous atmosphere and the decrease of the rate of photosynthesis with the rising oxygen concentration is studied. That the depression of the rate under the influence of oxygen actually can be used to obtain information on photooxidation processes will be shown in this paper. The second method introduced by van der Paauw (2) is a direct one and is the method used by us. Plants are irradiated in gaseous atmospheres containing oxygen but practically no

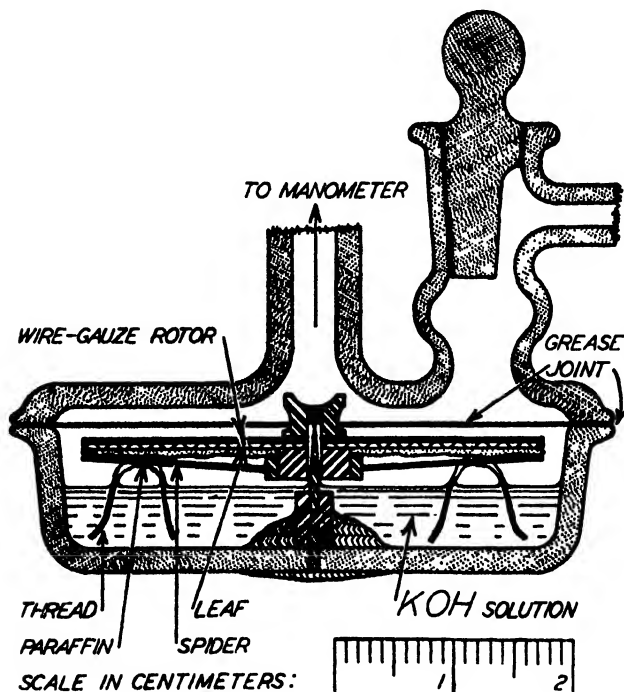


FIG. 1. A modified Warburg vessel in which a leaf is mounted on a rotor, with the lower surface containing the stomata just above a pool of KOH solution.

$\text{CO}_2$ , the  $\text{CO}_2$  produced by respiration and photooxidation having been removed as efficiently as possible by KOH solution. The influence of photosynthesis on the pressure readings thus becomes small under these conditions and the oxygen uptake under the influence of light can be measured manometrically. Fig. 1 shows the main part of the modified Warburg manometer used by us; some improvements and adaptations for the purposes of our experiments distinguish it from van der Paauw's original device.

A round piece of hydrangea leaf 3.7 cm. in diameter with a 0.6 cm. hole in its center was cut out with a punch and mounted under a wire gauze rotor with the underside of the leaf down. This rotor was supported on a jewel bearing by a needle so that the leaf was just a few millimeters above the surface of a layer of 10 per cent KOH

solution in the glass vessel of Fig. 1. Two paraffined threads looped over the rotor trailed in the KOH thus stirring it adequately. On top of the rotor was a magnetized iron strip which caused the leaf to rotate at a rate of 145 R.P.M. when a rotating magnetic field was applied from the outside.

The light source was a 2000 watt bulb with a spherical mirror behind it; the light was collected by a pair of 10 inch condenser lenses mounted together. An image of the condenser lens was focussed on to the bottom of the vessel of Fig. 1 contained in a glass-walled thermostat tank, with the aid of another large condenser lens and a mirror placed in the water. 2 inches of flowing water served as an infrared remover. Large Kolle culture flasks filled with  $\text{CuSO}_4$  or with  $\text{K}_2\text{Cr}_2\text{O}_7$  solution were used in special experiments as color filters. Calibrated wire screens were used to vary the light intensity.

(Later, the lens system was replaced by an elliptical mirror of 1 foot in diameter. This focussed the light of a 1000 watt projector bulb directly on the vessel without any lenses and gave a maximum intensity of about 80,000 lux.)

Practically all experiments were done with cuttings of hydrangea leaves. Other leaves when tested gave the same type of results. Attempts to use algae spread over the surface of moist filter paper were abandoned since the algae dried out too quickly under the influence of the strong light needed for our experiments.

Experiments with color filters showed that strong photooxidation is obtained with red or blue wavelengths, thus indicating that it is the chlorophyll which sensitizes this reaction as these are the wavelengths wherein chlorophyll absorbs most strongly.

Figure 2 *a* gives typical curves for manometer readings with pure oxygen in the dark and the light. The pressure decrease in the curves with the slopes  $\lambda = 7$  and  $\lambda = 8.5$  is produced by respiration, while the much steeper slope  $\lambda = 16.3$  is due to the combined effects of respiration and photooxidation. The slope is furthermore influenced by the fact that when illuminated a part of the respiration  $\text{CO}_2$  can be photosynthesized before it escapes out of the leaf and into the KOH solution. Further, the leaf becomes warm by illumination in spite of the circulation of the gas atmosphere in the thermostated vessel. This heating produces a rise in pressure which will be reversed the moment the light is turned off.<sup>1</sup> Generally, the pressure rise due to heating of the leaf at the beginning of an illumination is several millimeters of water greater than the pressure fall due to cooling at the end of an illumination period. An effort was made to determine whether this pressure difference could be used as a measure of the concentration of intermediate products of photosynthesis left over in the leaf from previous illumination periods in the presence of  $^3\text{CO}_2$ . These inter-

<sup>1</sup> It was not possible to calculate from the pressure changes above, the change in temperature of the leaf which occurs when the light is turned on. This temperature change was therefore measured with a thermocouple, but because of experimental difficulties these measurements were made only with non-rotating leaves. The temperature rise was  $1.3^\circ\text{C}$ . at the intensity most frequently used in our experiments and required about 5 minutes. The temperature dropped at an equal rate when the light was again extinguished. Of course, in a rotating leaf, the temperature change would not be as great nor would so long a time be required for the new steady state to be attained.

mediates would then be further reduced in producing oxygen under the influence of the illumination until their supply is exhausted. Since the observed pressure differences were only of the order of 1-4 mm., scarcely greater than the experimental error, we cannot be certain that the pressure changes observed were due to this effect. Certainly, it has no influence on the calculation of the rate of photooxidation, since for that purpose we have used only the slope of the curves after they had become linear. Usually, photooxidation is followed by a period of enhanced respiration. This period

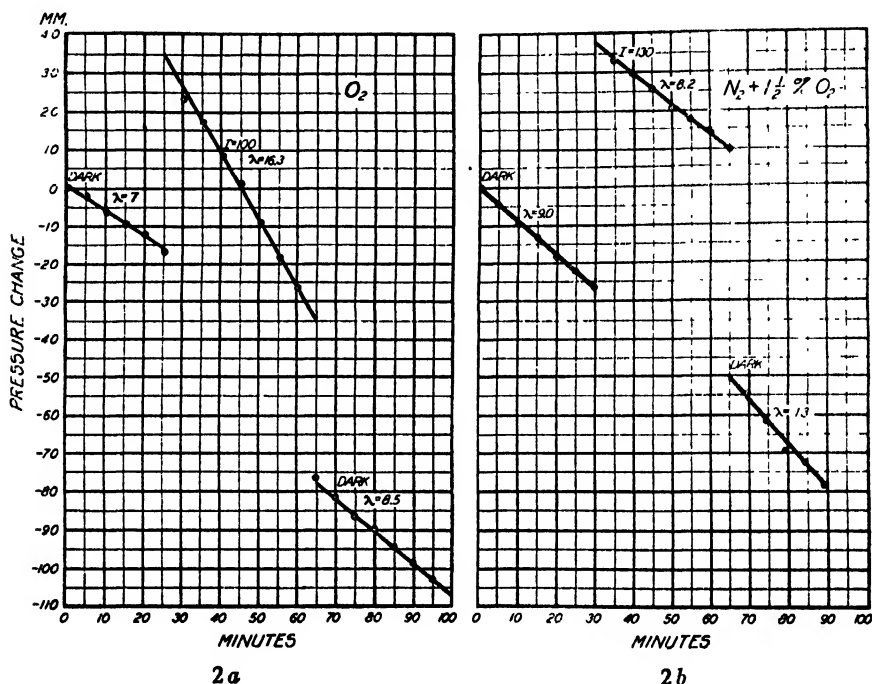


FIG. 2. Measurements of oxygen consumption in the light and dark in an atmosphere of oxygen as compared with measurements in nitrogen containing 1.5 per cent oxygen.

is not to be confused with the time necessary to reach thermal equilibrium. It takes but 2-3 minutes to balance the thermal effects whereas the enhanced respiration may last 10-30 minutes and even longer under certain conditions. The explanation of this was briefly mentioned in the introduction and will be discussed further below. Finally, one must take into account the fact that the pressure measurements are influenced by photosynthesis. Indeed, this effect is larger than we expected it to be under the conditions of our experiment. The photochemical reduction of  $CO_2$  formed by respiration was measured in an atmosphere of nitrogen containing just enough oxygen so that the rate of respiration is not limited by the  $O_2$  tension. In the case of hydrangea leaves about 2 per cent oxygen is enough as may be seen from Fig. 3, wherein the rate of respiration is plotted as the function of oxygen pressure.

Fig. 2*b* gives a set of curves as measured in  $N_2 + 1\frac{1}{2}$  per cent of  $O_2$  in the dark and in the light. It shows that the photooxidation is negligible under these conditions while photosynthesis compensates for a part of the respiration.

From the foregoing, it appears that since the rate of photooxidation in oxygen is calculated from three independent rate measurements, the final accuracy of the result is small. The precision is further reduced by the fact that the rate of photooxidation is not constant but decreases with time. The velocity of the decay depends upon the previous life history of the leaf, the light intensity, and the oxygen concentration. A leaf which has been well fed by photosynthesis or whose sugar supply has been enriched by immersing its stem into glucose solution shows a somewhat stronger photooxidation which also remains constant for a longer period. (In measuring the degree to which photooxidation of a given leaf depends upon external factors such as light intensity or oxygen

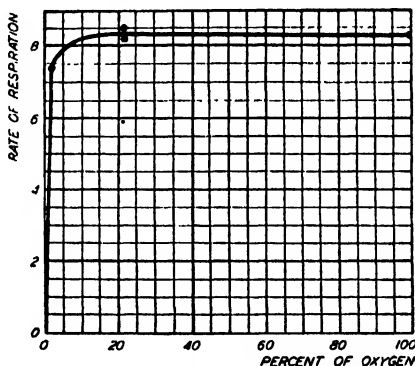


FIG. 3. Dependence of the respiration rate on the partial pressure of oxygen

concentration, one must be careful not to use too long irradiation periods. One can insure the absence of this error in the curves obtained by measuring the various points on the curve in a random order.)

The curve showing the rate of photooxidation as a function of oxygen concentration is of the same type in all leaves and is independent of their previous treatment. Fig. 4 presents an example.

The ordinate represents the rate of oxygen consumption by photooxidation corrected for respiration and photosynthesis; the abscissa is the percentage of oxygen contained in the atmosphere which was at normal pressure. For the reasons mentioned above, the accuracy is not great, but it is sufficient to determine the shape of the curve. The rate rises rapidly at first with the oxygen concentration. Later, the slope becomes smaller and finally one reaches  $O_2$  saturation; the rate becomes independent of further increase in the oxygen concentration. The saturation point is reached when about 60 per cent of the gaseous atmosphere is  $O_2$ .

Curves in which the rate of  $O_2$  uptake is plotted against the light intensity



reveal the fact that the rate rises less than linearly with the light intensity at least at high intensities. It was impossible for us to measure the shape of the curve accurately enough to determine whether the rate is proportional to the square root of the intensity. (At high intensities the rate of photooxidation declines too rapidly with time for precise measurement.) To establish beyond doubt that the deviation from linearity is not merely a deception the following procedure was chosen. The rate was measured in bright, intermittent light

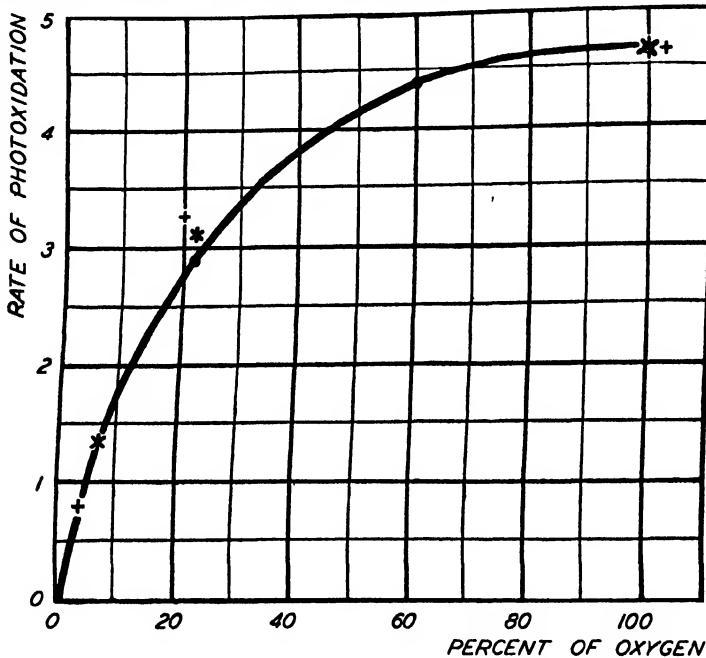


FIG. 4. Relative rate of different photooxidation processes plotted *versus*  $O_2$  pressure. Circles, our data for photooxidation in hydrangea leaves; crosses, Warburg's data for the decrease in rate of photosynthesis with  $O_2$  pressure; stars, Gaffron's data for the photooxidation of serum sensitized by adsorbed porphyrin.

and then immediately afterwards in continuous light of the same average intensity. Since the average illumination is the same in both cases there is no change in the temperature of the leaves and therefore no transient effect on the manometer readings such as would accompany a large increase or decrease in steady illumination. Any break in the pressure *versus* time plot must be attributed to a change in the rate of photooxidation. It is obvious that a change will occur only if the rate varies in a non-linear manner with the light intensity.<sup>2</sup>

<sup>2</sup> We furnished our brightest light with a large shutter pivoted on a bearing so that it could be lifted out of the light beam periodically and then dropped back. The shaker mechanism of the Warburg tank drove the shutter by a chain fixed with a stop so that the dark time was about 4 times the light period. The maximum intensity

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Table I shows some observations which indicate that the yield becomes lower at high intensities.

No particular effort was made to get exact data for the quantum yield of the photooxidation in leaves, since the effect varied so much with the conditions. But a comparison of the rates of photooxidation with the rates of photosynthesis which is obtained in these leaves makes it possible to estimate the order of magnitude of the quantum yield. Under favorable conditions it turns out to be  $\leq 1/100$  in an atmosphere of pure oxygen.

Experiments in which leaves were allowed to take up substantial amounts of sugar, by soaking the stem or the whole leaf in sugar solution, did not show much increase in photooxidation. However, if the corrections for respiration

TABLE I

*Photooxidation in Continuous and Intermittent Light of the Same Average Intensity*

Average rate per 30 min. period in mm./5 min.			Photooxidation corrected for respiration	
Dark	Continuous	Intermittent	Continuous	Intermittent
-3.8	-8.8		-4.0	
		-5.9		-1.1
	-6.8		-2.0	
		-5.6		-0.8
	-5.0		-0.2	
-4.8		-4.6		+0.2
	-5.5		-0.7	
	-6.5	-5.3	-1.7	-0.5

and photosynthesis were applied, the decay of photooxidation with time does appear to be slowed down.

A sugar-fed leaf irradiated in oxygen free from carbon dioxide shows a remarkable increase in its respiration, as may be seen by Table II.

In the case shown here, the respiration rose more than 100 per cent. The enhancement of the respiration lasts for hours provided the photooxidation period was long enough and the light during that period strong enough. Obviously the conclusion seems to be that under the influence of photooxidation, hexose is chemically changed into products which are more easily oxidized by the normal respiratory catalysts or are themselves autoxidizable. It is known

was 12.8 times the average intensity. The flashes came at the rate of 66 per minute. Now the average intensity of the alternate light and dark periods was measured by the increase in pressure (84.7 mm.) in the manometer vessel when a black paper was substituted for the leaf. It was found that screens transmitting 7.8 per cent of the continuous light made the intensity of the continuous light very nearly the same (85.6 mm.) as that of the integrated flashing light.

that in chemical oxidation processes hexose shows such behavior, but it is uncertain whether it is justifiable to compare such auto-oxidations *in vitro* with the respiration processes in the leaf.

Prolonged photooxidation damages the photosynthetic apparatus and finally kills the leaf, whereas with a short exposure to light and oxygen, in the absence of  $\text{CO}_2$ , the damage is reversible. This fact (which is in agreement with results of Myers and Burr (3) to be discussed later) was revealed in the following

TABLE II

Oxygen uptake in 10 minutes mm. Brodie solution	Respiration	Photoxidation (corrected)
	Before exposure in pure $\text{O}_2$ ...12.1	
		1st photoxidation period last- ing 25 min..... 8.4
	After 1st photoxidation period.....14.5	
	Returning after 2 hrs. in the dark to.....12.7	
		2nd photoxidation period last- ing 25 min..... 8.6
	After 2nd photoxidation period.....22.5	
	Constant over a period of 90 min.....22.5	
		3rd photoxidation period last- ing 25 min.....10.0
	After 3rd period.....24.0 (Measured 30 min.)	

way: leaves were exposed to light in the presence of 1 per cent  $\text{CO}_2$  and the photosynthetic rate was measured. Then they were subjected to photoxidation for a short time by exposure in  $\text{CO}_2$ -free oxygen (generally about 20 minutes) and afterwards photosynthesis was again measured. The photosynthetic activity recovered slowly. The time necessary for the recovery depends on the illumination; intensities just great enough to produce saturation of photosynthesis give much quicker recoveries than the smaller ones.

Fig. 5 gives an example of such measurements. Before the treatment the induction loss is unobservably small (upper curves); after treatment (lower curves) the induction period lasts about 15 minutes but the rate comes back to its old value. Recovery of photosynthesis in the region where the damage

is reversible can be accomplished not only by photosynthetic activity but also by subjecting the leaf to long dark periods. In some cases where photooxidation was followed by a long dark period we observed that on reillumination, photosynthesis was resumed with an induction loss no greater than the usual one in normal untreated leaves.

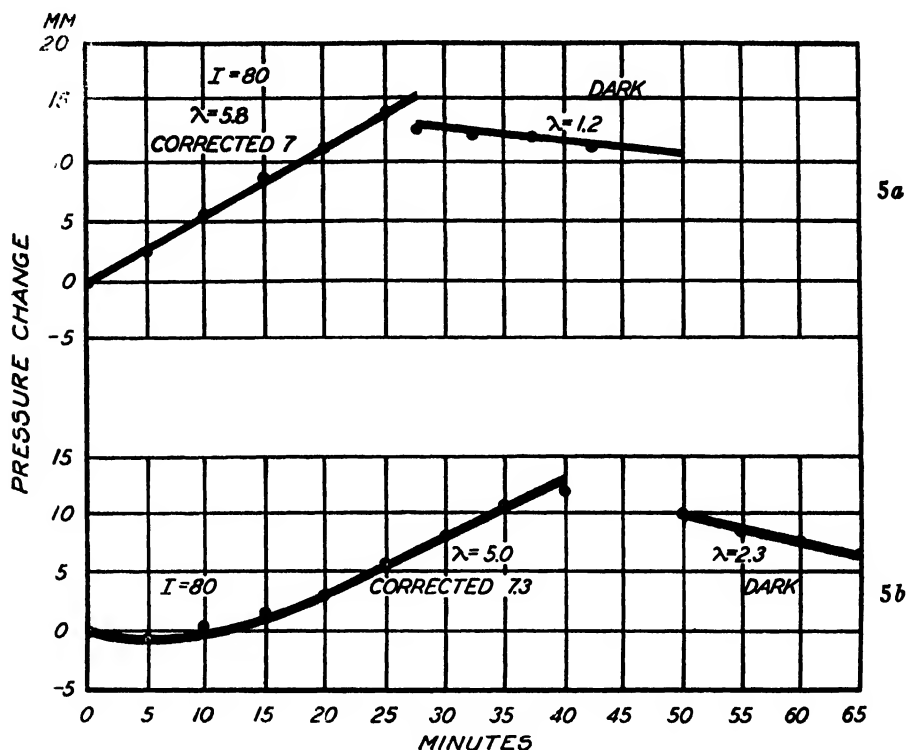
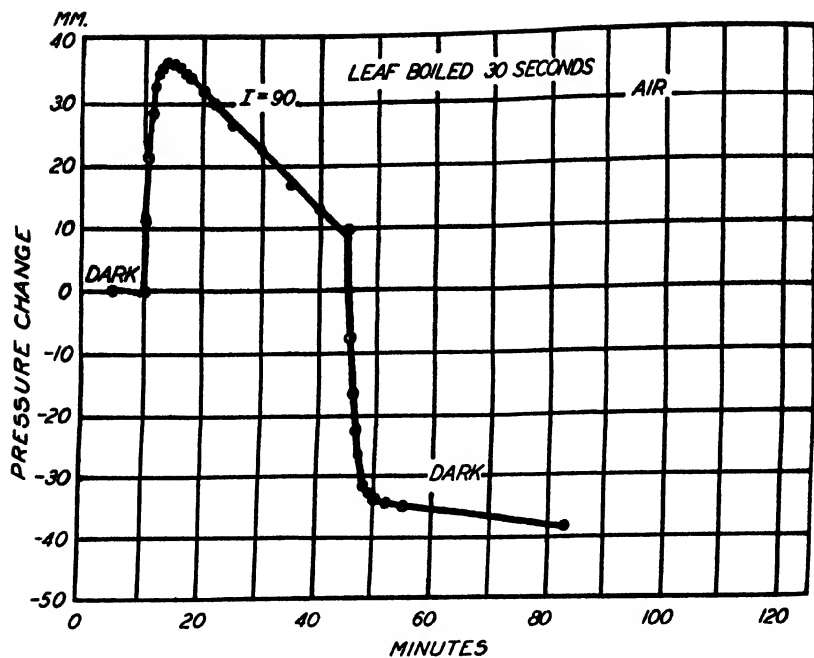


FIG. 5. Photosynthesis measured in the presence of sufficient  $\text{CO}_2$  both before (5 a) and after (5 b) a photooxidation period.

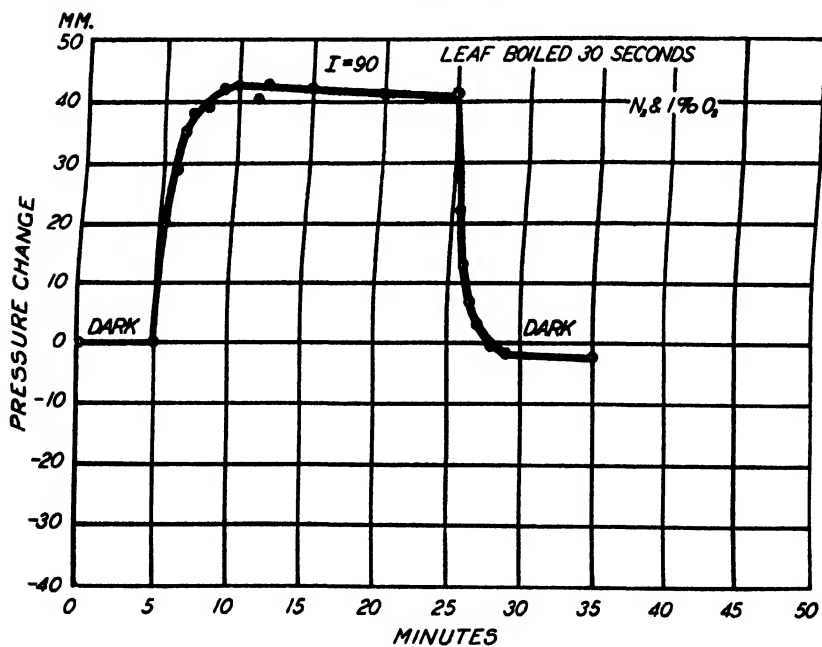
A series of experiments was made to find out whether photooxidation in leaves depends on the action of heat-labile enzymes. For that purpose leaves were killed by submerging them for 20 seconds to 5 minutes in boiling water. The results show that photooxidation takes place in dead leaves just as well as in live ones. (Compare Fig. 6a and b.) Also, the dependence of the rate of photooxidation upon light intensity is unaltered within the accuracy of the experiments. The rate of photooxidation in the boiled leaves seems a little smaller than in the fresh ones.

Finally, some experiments done in this laboratory<sup>3</sup> may be mentioned which

<sup>3</sup> The experiments were carried out by T. Puck and C. S. French.



6a



6b

FIG. 6. Measurements of oxygen consumption in a boiled leaf in the light and dark in an atmosphere of oxygen (6 a) as compared with similar measurements in nitrogen containing 1.5 per cent oxygen (6 b).

show that plant juices containing whole chloroplasts or only their macerated contents (mostly consisting of chlorophyll adsorbed on its native protein) show photooxidation. The rate of oxygen consumption is smaller than in leaves, but can be made even stronger than in leaves by addition of easily oxidizable substances like ascorbic acid. Ascorbic acid is also oxidized in the dark but at a much lower rate, so the rate of photooxidation can be calculated from the enhancement of the oxygen uptake during the illumination period. The experiments are in accordance with the observations made by French (4) in water extracts of purple bacteria.

#### *Comparison of the Results with Older Observations*

It has been known for a long time as mentioned in the introduction, that photooxidation processes occur in plants. Noack (5) showed already in 1923 that substances whose oxidation products can be recognized by their color can be photo-oxidized in plants into which they are artificially introduced. The same author stated that  $O_2$  absorption is increased in leaves illuminated for 84 hours in absence of  $CO_2$ . Van der Paauw whose method of observation is comparable to ours undoubtedly observed photooxidation as well as its enhancing influence on respiration in the dark directly following an illumination period. Van der Paauw himself comes to the conclusion that in his observation the normal tissue respiration is enhanced by "plasmogeneous influences." We think that his point of view is not tenable for the following reasons: as was discussed above, the  $O_2$  uptake depends on the partial pressure of oxygen in a manner entirely different from that of respiration. Furthermore, killed leaves which do not respire and plant juices show a comparable  $O_2$  uptake in the light. The same remarks apply to the conclusion which Shri Ranjan (6) draws from similar observations. Likewise, the papers of Föckler (7) and Föckler and Montfort (8) are not convincing in their conclusions based on analogous observations. In respect to these papers we agree with Gaffron's (9) criticism.

The work of Mevius (10) is related to photooxidation in plants exposed to light in a  $CO_2$ -free atmosphere. This author studied the behavior of leaves of higher plants still connected with the intact plant. A part of the plant was exposed to light in a  $CO_2$ -free atmosphere while other parts of the plants were irradiated in normal air and were therefore able to photosynthesize. Mevius found that leaves could stand long exposures to light in the  $CO_2$ -free atmosphere without observable damage provided that the photosynthesizing leaves supply enough food for the exposed leaves. If that flow of nourishment stopped or became too small, starch and sugar were consumed and the leaves turned yellow with the destruction of proteins.

All other conclusions on the occurrence of photooxidation are based on observations of the rate of photosynthesis. The most important contributions of this type are those of Warburg (1) and of Myers and Burr (3). Warburg's

first observations of the influence of oxygen concentration on the rate of photosynthesis yielded the result, which has often been confirmed since, that even in the presence of sufficient  $\text{CO}_2$  the rate of photosynthesis at light saturation depends strongly on the oxygen concentration. Under some conditions the rate may be reduced to 60 per cent if an atmosphere of  $\text{N}_2$  (containing enough oxygen to saturate respiration) is replaced by an atmosphere of pure oxygen. Sometimes, on the other hand, the retarding influence of oxygen is quite small. It is known that the difference in behavior depends on internal factors such as food concentration and respiration. At low intensities neither Warburg nor others (compare McAlister and Myers (11); Myers and Burr) found any influence of the oxygen concentration on the rate of photosynthesis.

An exception is found in the results of Katz and Wassink and coworkers (12), who observed that at low intensities as well as at high ones, a diminishing of photosynthesis occurs with increase in the oxygen concentration.

Most authors do not relate these strong influences of oxygen concentration on the rate of photosynthesis to photooxidation, but a comparison of Warburg's results with ours unmistakably reveals an intimate connection between these effects. The most direct proof is that the variation of the photosynthesis rate with oxygen pressure follows the same sort of curve as does our photooxidation data. Moreover, this curve is similar to the one Gaffron (13) observed in 1926 for the photooxidation processes occurring in an aqueous solution in which porphyrins or chlorophyll were adsorbed at the surface of the proteins. In Fig. 4 are recorded Gaffron's observation (marked with stars) together with those of Warburg (marked with crosses), and our own. The fact that the significant variations occur in the same range of oxygen pressures for all three sets of observations is a strong indication that the phenomena are related. We see a further connection in the fact that after a long irradiation the difference between the rates of photosynthesis in  $\text{N}_2$  and  $\text{O}_2$  respectively is greatly reduced, just as photooxidation in leaves goes down with the irradiation time.

A great quantitative difference exists, however, between the results of the direct and the indirect methods of photooxidation measurement. The diminishing of the rate of photosynthesis by oxygen is usually 10 to 20 times greater than the rate of photooxidation itself; one must therefore assume that the direct uptake of oxygen by photooxidation is small compared to the indirect influence on photosynthesis. That is just the same conclusion to which Myers and Burr (3) were guided by their observations of the influence which extremely high light intensities have on the photosynthetic rate in *Chlorella*. These authors did not vary the oxygen concentration but irradiated the algae in the presence of air containing  $\text{CO}_2$  and in some cases, cyanide as well. They observed normal saturation curves forming a flat plateau, but when the light intensity surpassed a critical value the rate started to decrease and continued to do so with further increase of light intensity. At the very highest values of the

light intensity the oxygen production ceases entirely and is replaced by an oxygen consumption which is greater than the normal respiration. The critical intensity at which the influence of the photooxidation becomes discernible depends upon conditions inside of the plants (concentration of  $\text{CO}_2$ , food, presence or absence of cyanide, etc.). Franck and Gaffron (14) have already pointed out that the critical intensity apparently coincides with the intensity region at which the photosynthetic apparatus begins to become depleted of  $\text{CO}_2$  and hence of intermediates of photosynthesis. (See the theory of Franck and Herzfeld (15) and fluorescence experiments of Franck, French, and Puck (16).) A catalyst (called catalyst A) responsible for the formation of the compound  $\text{CO}_2$ -acceptor-molecule becomes unable to replace this substrate rapidly enough at that intensity. Further, the greater the amount by which the light intensity surpasses the saturation intensity, the more rapidly is this substrate destroyed by dissociation processes. Each shift of the region in which depletion of this substrate occurs (for instance, by addition of cyanide) produces a corresponding shift of the critical region in Myers and Burr's experiments. It is obvious that by a depletion of the photosynthetic apparatus of intermediates the bulk of the chlorophyll becomes available as a sensitizer of photooxidative processes and so produces a sharp rise in the yield of photooxidation. Myers and Burr (3) reject the idea that the consumption of  $\text{O}_2$  by photooxidation at the highest intensities would overcompensate photosynthesis. They rather assume that photooxidation produces an inhibitor of photosynthesis. The main reason for this hypothesis is the fact that the photosynthetic activity is found to be reduced or entirely stopped after a photooxidation period when tested with light intensities so low that photooxidation no longer plays a rôle. The rate of photosynthesis recovers if the photooxidation period was not too strong and too long. These results of the indirect method agree entirely with ours measured directly. By too intensive irradiation the plant will be killed and bleached.

It does not seem necessary to enter into a discussion of further similar papers since they are mostly in accordance with the facts already discussed. (For references, compare, for instance, Myers and Burr (3), and Franck and Gaffron (14).)

#### THEORETICAL DISCUSSION

The experimental data discussed above prove beyond doubt that photooxidation processes occur in plants even under normal conditions. It is a photochemical process taking place together with photosynthesis, but with a much smaller yield. Normally, it does not attack vital parts of the living plants but food or intermediate products of the plant metabolism. As long as the photosynthetic apparatus is connected with intermediates of photosynthesis, the photooxidation is small, but it nevertheless reduces the rate of photosynthesis



strongly though indirectly. If by carbon dioxide limitation or some denudation process the concentration of the photosynthetic intermediates becomes small, photooxidation will become dangerous since vital parts of the plant will now be attacked. The plant can be destroyed by photooxidation, a phenomenon described by botanists as solarization of plants.

The photooxidation processes in plants take place without enzymatic dark reactions (see the experiments with boiled leaves) but probably consist rather of a photochemical process sensitized by the plant pigment, chlorophyll. The characteristics of this process are just the same as the ones observed in photooxidation processes *in vitro* sensitized by chlorophyll or porphyrins adsorbed by proteins in aqueous solution.

Two questions deserve a fuller discussion, the first concerns the process by which photooxidation acts as an inhibitor of photosynthesis and the second deals with the general chemical kinetics of photooxidation processes induced by chlorophyll adsorbed on protein surfaces.

According to Franck, French, and Puck) 16), one must assume that during the induction period of photosynthesis an excess of photo-peroxides can oxidize certain metabolites and that the resulting products in turn are capable of inhibiting photosynthesis by poisoning the catalyst responsible for photosynthesis saturation (called catalyst B). To explain the inhibition by photooxidation one need only make the hypothesis that this process results in the formation of the same or a similar inhibitor. It offers a natural explanation for the fact that the limiting influence of a high oxygen concentration vanishes at low light intensities. At these low intensities a great part of catalyst B can be inactivated by the inhibitor without influencing the rate of photosynthesis.

The dependence of the magnitude of the oxygen influence on internal factors can be understood by the fact that the concentration of the metabolic product (which is attacked by photooxidation) varies considerably in leaves. The reversal of the inhibition can occur in the dark since the inhibiting oxidation product is consumed by respiration.

We now inquire as to why the recovery time of photosynthesis is much longer (several hours) than the lifetime of the inhibitor (about 1 second, see Franck, French, and Puck). The abnormally long induction periods of photosynthesis and the still longer dark recovery periods required after photooxidation have to be explained in a different manner. We believe that they both are caused by the denudation of the photosynthetic apparatus of intermediates. A normal rate of photosynthesis is only possible if the supply of intermediates is not too small and if the different intermediates all have the same concentration. If intermediates are lacking at the beginning of an irradiation period in the presence of carbon dioxide, it takes some time to build up equilibrium concentrations by photosynthetic activity. Until that is accomplished, the photosynthetic rate is subnormal. The time observed for the duration of the induction periods (at different light intensities) after an exposure in a carbon

dioxide free atmosphere is of the expected order of magnitude. According to Franck and Herzfeld (15) the intermediates can also be very slowly restored in the dark by a reversal of photosynthesis (not identical with normal tissue respiration). We believe that this process is responsible for the recovery in the dark.

We will now discuss the chemical kinetics of photooxidation sensitized by chlorophyll adsorbed on protein in an aqueous solution. The fact that the reaction becomes oxygen-saturated at an oxygen concentration which is many thousand times greater than the one which saturates photooxidation processes sensitized by chlorophyll in organic solutions is of special interest. This means that in the aqueous solutions oxygen has to react with a very unstable molecule. One can calculate from the oxygen concentration at the saturation pressure that the lifetime is equal to or somewhat greater than  $10^{-8}$  to  $10^{-9}$  seconds. The obvious explanation that the molecule in the unstable state is the chlorophyll molecule excited by light absorption must be rejected for two reasons. In the first place the lifetime of the excited chlorophyll molecule (compare Franck and Herzfeld (17)) in the leaves is still 10 to 100 times smaller than the above value and secondly, the fluorescence of the chlorophyll in leaves is not, or at least is practically not, quenched by an atmosphere of oxygen. To explain the behavior of the chlorophyll fluorescence in relation to photooxidation processes in organic solutions, Franck and Livingston (18) had to introduce the hypothesis that chlorophyll can be transformed by light absorption processes into an unstable tautomeric configuration with a lifetime much longer than  $10^{-8}$  seconds. But according to Livingston's (19) observations this lifetime will be shortened by impacts with molecules of many organic substances which catalyze back reactions leading to the stable modification of chlorophyll. In the present case the chlorophyll is in permanent contact with the protein, a fact which easily can account for the reduction of this lifetime to the desired value. We, therefore, propose that impacts between the chlorophyll tautomer and oxygen are also responsible for the photooxidation. By the reaction of the unstable tautomer with oxygen, monodehydrochlorophyll and  $\text{HO}_2$  are formed (as in organic solutions—compare Weiss (20) and Franck and Livingston (18)). The radical  $\text{HO}_2$  is able to oxidize organic matter but its oxidation reactions have to compete with back reactions in which the stable modification of chlorophyll and molecular oxygen are restored. In very dilute, true solutions the chance for back reactions is small but since in our case the chlorophyll is adsorbed in a high concentration on complicated interfaces of a protein, the back reactions become very significant. The  $\text{HO}_2$ , especially at higher light intensities, has ample opportunity to collide with monodehydrochlorophyll before it can attack the substrate to be oxidized. Calculations yield the result that under the conditions mentioned the quantum yield must be small for small concentrations of the oxidizable substrate and that the oxygen consumption will rise with the square root of the light intensity. The first result is in

accordance with our observations and the last one is at least not in contradiction to the results here presented, within the limits of error of the experiments.

#### SUMMARY

1. Photoxidation in leaves is measured by exposing them to light in an atmosphere free from carbon dioxide but containing varied percentages of oxygen.
2. Photoxidation is observed in living leaves as well as in dead ones and in plant juices. Its rate is only slightly enhanced by feeding the leaves with sugar, but the respiration (autoxidation) becomes considerably enlarged during the exposure and the following dark period.
3. The rate of photoxidation rises slower than linearly with light intensity; its dependence upon oxygen pressure has the character of a saturation curve. Oxygen saturation occurs at about 6/10 of an atmosphere of oxygen. A similar dependence on oxygen pressure has been observed by Gaffron for photoxidation *in vitro* sensitized by chlorophyll adsorbed on proteins and by Warburg for the depression of the saturation rate of photosynthesis.
4. The influence of photoxidation on photosynthesis and the chemical kinetics of photoxidation are discussed.

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## THE MAGNETIC BEHAVIOR OF CATALASE

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Pauling and Coryell's (1) measurements of the magnetic properties of hemoglobin and several of its derivatives have revealed that observations along these lines are capable of contributing a great deal to the elucidation of the chemical structure of such compounds. Hemoglobin has turned out to be one of the strongest paramagnetic substances whereas oxyhemoglobin is not paramagnetic at all, indicating a profound change in the nature of the chemical bonds between iron and the attached groups as a result of the combination with molecular oxygen. Such a surprising result suggests extending this study to other heavy metal complexes of biological interest. It is the task of this paper to extend such measurements to catalase. Here, the task is very much more difficult than for hemoglobin. In addition to the difficulty of obtaining enough material, there is the risk of denaturation on thorough drying. It is not advisable to investigate the dried crystals of catalase. It is not feasible, either, to attempt measurements in solution since the solubility is too small within the permissible pH range. So one has to resort to investigating suspensions of the wet crystals. Fortunately, such a suspension in a suitable phosphate buffer is stable enough and sedimentation is negligible. However, a further difficulty remains, namely, that catalase has nearly four times the molecular weight of hemoglobin. Its iron content is 0.1 per cent approximately; *i.e.*, about one quarter that of hemoglobin. So it is impossible to obtain solutions or even suspensions comparable in iron content per cubic centimeter with that of readily obtainable concentrations of hemoglobin solutions.

Supposing the susceptibility per gram-atom Fe to be the same as in hemoglobin (in reality it is even smaller), a solution or suspension of catalase would produce only one quarter of the pull in a magnetic field of that of a hemoglobin solution of equal concentration. For this reason the apparatus used by Pauling and Coryell is scarcely sensitive enough. Recently a modification of the method has been developed in this laboratory which was originally used for the assay of free semiquinone radicals in solution. It is adequate also for the present task. This method is, like that of Pauling and Coryell, essentially Gouy's method converted into a differential method in order to obtain higher sensitivity. Some technical details have been published recently and since the method

is being still further refined, it may suffice to describe its principles roughly, postponing the ultimate details until some later time.

The solution to be measured is filled into the upper compartment of a cylindrical double vessel (Fig. 1), the lower compartment of which contains either water or some solution of suitable susceptibility which is never changed during a series of measurements. Only the contents of the upper compartment are

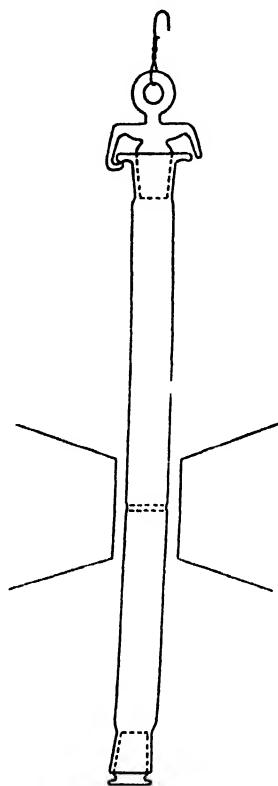


FIG. 1

varied. This vessel is suspended from one pan of a balance so that the diaphragm of the vessel is located between the centers of the pole pieces of an electromagnet. When the magnetizing current is switched on, the pull of the magnetic field on the upper and the lower compartment of the vessel is in opposite directions, and only the difference of pull is measured. This increases the sensitivity of the Gouy method very considerably. This differential method was first used, at least for solutions, by Freed and Kasper (2). The pull is measured in the following manner: The semi-micro, magnetically damped balance is equipped with a scale at the pointer with 200 divisions, each corresponding to

about 1/100th of a milligram, which is read through a microscope. What is observed is only the maximum deflection from the resting position brought about by abruptly switching on the current. Such readings are reproducible to  $\pm 2$  lines of deflection, and a series of ten readings gives a reliable average value. The current intensity is adapted to give a deflection of 20 to 120 lines. It was ascertained that deflections within all current intensities used, namely up to 10 amperes, are proportional to the square of the amperage. All readings are recalculated accordingly to 10 amperes.

The significance of each line of deflection, in terms of susceptibility, depends on the diameter of the vessel, the distance of the pole pieces, and the amperage. For each vessel, with given pole distance, the "vessel constant," *i.e.* the factor by which the number of lines of deflection must be multiplied to obtain susceptibility in c.g.s. units, is determined as follows. Keeping the contents of the lower, compensating compartment constant, the pull is measured first with air in the upper compartment, then with water in it. The difference corresponds to a change of susceptibility  $= 0.74 \times 10^{-6}$ , of which  $0.2 \times 10^{-6}$  is that of air and  $-0.72 \times 10^{-6}$  that of water. Now,  $0.74 \times 10^{-6}$  divided by the difference of the numbers of lines of deflection, each recalculated for 10 amperes, for air and for water, is the vessel constant.

In an actual experiment, the upper compartment is filled with the solution, then with the buffer or solvent of the same composition, but not containing the dissolved (or suspended) substance to be investigated. The difference between the two values, expressed in lines of deflection, multiplied by the vessel constant, yields directly the increment of susceptibility due to the substance being measured. Small corrections may be necessary; however, they are quite irrelevant for the present purpose and may be omitted from this outline of the method. The increment in susceptibility divided by the grams of the specific substance in 1 cc. gives the susceptibility per gram of the substance. Multiplying this figure by 56 gives  $\chi$ , the susceptibility of 1 g.-atom of iron, in the form of catalase. Herefrom the magnetic moment, per gram atom of iron,  $\mu$ , is obtained by the equation

$$\mu = 2.84 \sqrt{\chi \cdot T} \quad \text{Bohr magnetons}$$

where  $T$  is the absolute temperature at which the measurement has been made.

To check the method, the magnetic moment of  $K_3Fe(CN)_6$  was determined in a 1/250 M solution, another in a 1/30 M solution, with the results: 2.44 resp. 2.25 Bohr magnetons using 1.7 cc. of the solution to fill the vessel. The first of these measurements is based on an amount of about 1.7 mg. of the substance, giving a differential deflection compared with pure water of about 25 lines. The acknowledged value is 2.33 magnetons; the agreement is satisfactory in spite of the minute amount of substance used. In one single experiment with a so-

lution of crystallized guinea pig ferro-hemoglobin in solution we found  $\mu = 5.25$  per g-atom of hemoglobin-iron, as compared with 5.46 according to Coryell and Pauling.

The experiments with crystallized catalase were performed with a preparation made from beef liver according to the method of Sumner and Dounce (3), recrystallized once. The thin flat platelets were suspended in a mixture of equal volumes of Sørensen's phosphate buffer pH 7.4, and water. This buffer was suitable for keeping the crystals in suspension without any appreciable sedimentation occurring. The iron content of the dry catalase was 0.0918 per cent. The iron content of the final suspension per cc. was 0.0586; 0.0568; 0.0593; 0.0604; 0.0606; 0.0595; the average 0.0596 mg. This analysis was made colorimetrically with the *o*-phenanthroline complex (Hummel and Willard (4)) on samples containing 0.03 to 0.1 mg. Fe. The samples of the catalase suspension were digested with 1 cc. concentrated  $\text{H}_2\text{SO}_4$  and 0.3 to 0.5 cc.  $\text{HNO}_3$  and two drops of 70 per cent perchloric acid in a 100 cc. Kjeldahl flask. Blanks were determined correspondingly. The following remarks may be helpful. Prolonged heating after  $\text{SO}_3$  fumes start to come off should be avoided to prevent as much as possible the formation of pyrophosphate and anhydrous ferric sulfate. After the digestion is complete, 10–20 cc. of water are added and the solution kept boiling slowly for an hour to decompose any pyrophosphate which would seriously interfere with the later development of the color. Even after this treatment, the full development of the color with phenanthroline takes some time. If the solution, before adding phenanthroline, is allowed to stand for 24 hours, the full color will be developed in several minutes. The final result, whether attained the one way or the other, is the same. The interference of pyrophosphate is serious if not well taken care of, but can be entirely overcome once the source of error is recognized.

The suspension, kept on ice, was used for several magnetic measurements over several days. The differential pull under the most favorable conditions, at 10 amperes, is about  $\frac{1}{4}$  to  $\frac{1}{3}$  of a milligram. This ought to be sufficient to yield fairly reproducible results. In fact, there is a rather large standard deviation of the results. The authors attribute this fact mainly to warm and humid air conditions which are not favorable for precise weighing. A repetition, with a further refinement of the method, is planned for the near future. The present results are shown in Table I.

The result is: the magnetic increment per cc. of the suspension, due to the presence of 0.0596 mg. of Fe, is  $(+0.094 \pm 0.017) \times 10^{-7}$ . Hence, the susceptibility per g-atom Fe is  $8830 \times 10^{-7}$ . Herefrom the magnetic moment results as follows

$$\begin{aligned}\mu &= 2.84 \sqrt{8830 \times 10^{-7} \times (273 + 27)} \\ &= 4.64 \text{ Bohr magnetons} \pm 0.3\end{aligned}$$

The accuracy of the figure can certainly be improved by further refinements of the method, but even so, the result lends itself to a fair comparison with hemoglobin compounds as obtained by Pauling and Coryell, of which we mention only

Ferro-hemoglobin, Bohr magnetons per Fe atom.....	5.46
Oxy-hemoglobin.....	0.0
Ferri-hemoglobin.....	5.8
Ferri-hemoglobin hydroxide (alkaline methemoglobin).....	4.47

TABLE I

Various cylindrical, double vessels were used, of 6 to 8 mm. outer diameter, and 10 to 13 cm. length of each compartment. The column "Vessel constant" shows the change of volume susceptibility corresponding to a change of one line of differential deflection, according to the calibrations for the particular vessel. All measurements were made at a room temperature of 26 to 27°C. The column "Buffer" gives the deflections in lines, when a phosphate buffer of the same composition, without catalase, was measured. The column " $\chi$ " indicates the excess of volume susceptibility of the catalase suspension over that of the corresponding buffer. This is always a positive number, showing that catalase is paramagnetic. No corrections for any change in diamagnetic effect are applied. They are not worthy of consideration at the present state of refinement of the method.

	Buffer	Catalase	Difference	Vessel constant $\times 10^{18}$	" $\chi$ " $\times 10^6$
1	-54	-25	+29	4.36	0.126
2	-54	-33	+21	4.36	0.092
3	-84	-41	+43	2.89	0.123
4	-91	-48	+43	2.89	0.123
5	+16.7	+43.5	+26.8	3.15	0.086
6	+15	+39.5	+24.5	3.15	0.077
7	+5	+26.5	+21.5	3.18	0.069
8	+25	+45.8	+20.6	3.18	0.066
9	-18	+18	+36	2.55	0.0915
Average.....					0.094
Probable error.....					$\pm 0.017$

The magnetic moment of catalase is distinctly smaller than that of hemoglobin and resembles most that of alkaline methemoglobin.

It is suggestive to investigate also various reaction products of catalase. At the present stage of the investigation we do not wish to give accurate figures yet, but can state that addition of  $\text{Na}_2\text{S}_2\text{O}_4$  does not change the susceptibility, which seems to be a corroboration of Keilin's statement that catalase is not reduced by  $\text{Na}_2\text{S}_2\text{O}_4$ ; and that  $\text{NaCN}$ , and also  $\text{NaOH}$ , diminish the susceptibility. Since we are working with a suspension, not a solution of the substance, we hesitate to interpret these results for the time being and refrain from giving figures.



## CONCLUSION

The magnetic moment of catalase is  $4.6 \pm 0.3$ , nearly the same as that of ferric hemoglobin hydroxide. If the moment be due to electron spin alone without orbital contribution, 3 free electrons would give rise to 3.9 magnetons.

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# THE VISUAL SYSTEM AND VITAMINS A OF THE SEA LAMPREY

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What is known at present of the distribution of visual systems suggests that the rhodopsin-vitamin A<sub>1</sub> cycle is the primitive vertebrate type. The marine elasmobranchs, all marine teleosts except the Labridae, and all terrestrial vertebrates so far examined possess this system alone (Wald, 1938-39). The eyes of squid and of crabs also contain exclusively components of this system (Wald, 1941). The porphyropsin-vitamin A<sub>2</sub> cycle seems to be a comparatively recent development, associated with the evolutionary migration of teleost fishes into fresh water (Wald, 1938-39; 1941-42).

This appraisal of the situation can be tested critically in the cyclostomes, for it implies that these animals, though all freshwater in origin, possess only the rhodopsin-vitamin A<sub>1</sub> system. The cyclostomes—with the closely related myxinooids—are the most primitive living vertebrates, surviving members of the ancient class or subphylum Agnatha, believed to have pursued an independent evolution since earliest vertebrate origins. All of them are fresh-water or anadromous, but this property does not distinguish them from the supposedly freshwater vertebrate ancestors (Smith, 1932; Romer and Grove, 1935). If it is true that the porphyropsin-vitamin A<sub>2</sub> cycle is a specifically teleost innovation, the visual systems of cyclostomes should contrast sharply with those of freshwater and anadromous bony fishes.

In the present experiments this matter is examined in the sea lamprey, *Petromyzon marinus*, an anadromous cyclostome. The life history of this animal has been described in detail by Gage (1927). It spawns in freshwater streams in the late Spring, and dies shortly thereafter. The so called ammocete larvae are blind, and differ profoundly in structure from the adults. They spend 4-5 years in the sand or mud bottoms of the parent stream, feeding upon microorganisms. At a final larval length of 5-7½ inches they metamorphose to the adult form and usually migrate to the sea. Here they appear to feed primarily upon the blood of fishes to which they attach by suction through their disc-shaped mouths. The marine phase lasts 1½-3½ years, and is terminated by the return to fresh water to spawn. Like anadromous teleosts, sea lampreys can spend their entire life cycle in fresh water without detriment.

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The lampreys used in the present experiments were sexually mature animals, about 2 feet in length, taken from brackish tidal waters of the Exeter River in New Hampshire during the spawning run. The tissues were dissected out on the river bank and transported in dry ice to the laboratory. Through the kindness of Dr. E. J. W. Barrington I have been able also to examine the livers of late larvae of land-locked *P. marinus* taken from streams about Lake Oneida in New York State.

### *Eye Tissues*

The retinas dissected from highly light adapted animals in broad daylight were completely colorless. Under similar circumstances fish and amphibian retinas usually adhere firmly to the underlying pigment epithelium. The lamprey retina, however, readily comes away entirely free of pigmented tissue. This peculiarity is probably associated with the reported absence of pigment migration and photomechanical changes in *P. marinus* (Walls, 1935).

Batches of 40-60 retinas were extracted by shaking exhaustively with chloroform. The extract was saponified in 6 per cent KOH in methanol under a stream of nitrogen. The non-saponifiable fraction was taken up in benzene, transferred to 0.3 cc. of chloroform, and tested by mixing with 3.2 cc. of antimony chloride reagent. A spectrum of the resultant blue test solution is shown in Fig. 1.<sup>1</sup>

Such spectra are dominated by the high band at 692-696  $m\mu$  which characterizes vitamin  $A_2$ . A broad inflection at 620-640  $m\mu$  includes the 645  $m\mu$  "hump" which always accompanies the vitamin  $A_2$  band, and a low absorption due to vitamin  $A_1$ . The ratio of the vitamin  $A_2$  absorption at 696  $m\mu$  to that of vitamin  $A_1$  at 618  $m\mu$ , computed as described elsewhere, is 89:11 (cf. Wald, 1938-39).

The remaining tissues of the fundus, including the pigment epithelium, choroid and sclera, also contain a great predominance of vitamin  $A_2$ . The antimony chloride reaction with an extract of these tissues, prepared exactly like that of the retinas, is shown in Fig. 2. The ratio of vitamin  $A_2$  to  $A_1$  absorption in this preparation is 82:18. In another experiment it was 77:23. The amount of vitamins A in these tissues is about twice that in the retina.

The lamprey retina contains, therefore, not the vitamin  $A_1$  expected by virtue of its primitive and isolated phylogenetic position, but predominantly vitamin  $A_2$  like an anadromous teleost. The force of this comparison was emphasized by a curious circumstance. Our lampreys were accompanied

<sup>1</sup> All spectra shown in this paper are original recordings drawn with Hardy's photoelectric spectrophotometer at the Massachusetts Institute of Technology. The results are expressed as per cent absorption,  $1 - I/I_0$ , in which  $I_0$  is the incident and  $I$  the transmitted intensity. Before they can be used to compute relative concentrations they must be converted to extinction or optical density,  $\log I_0/I$ .

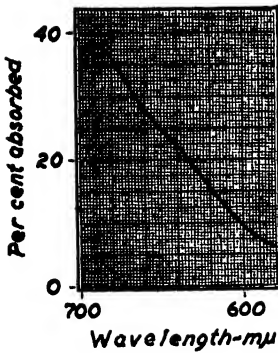


FIG. 1

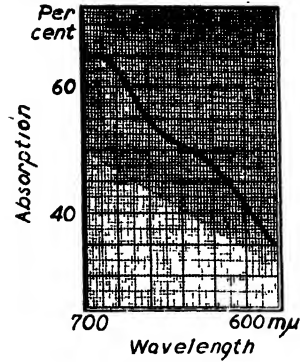


FIG. 2

FIG. 1. Spectrum of the antimony chloride reaction with an extract of lamprey retinas. A high band at 692  $m\mu$  due to vitamin  $A_2$  and a low absorption in the region of 618  $m\mu$  due to vitamin  $A_1$  are present in the proportion 82:11. The total non-saponifiable oil from 40 retinas, dissolved in 0.3 cc. chloroform, had been mixed with 3.2 cc. of antimony chloride reagent and the spectrum measured in a layer 1 cm. in depth.

FIG. 2. Spectrum of the antimony chloride reaction with an extract of lamprey pigment epithelia, choroids and sclera, prepared as in Fig. 1. Both vitamin  $A_2$  and  $A_1$  absorptions are present in the ratio 82:18.

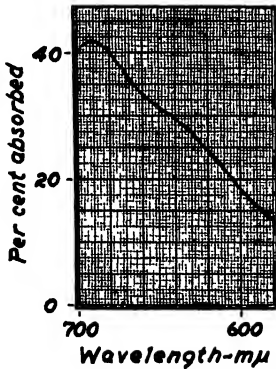


FIG. 3

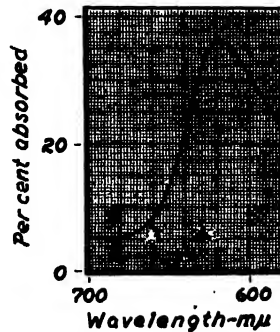


FIG. 4

FIG. 3. Spectrum of the antimony chloride reaction with an extract of retinas and pigment epithelia from the alewife, prepared as in Fig. 1. Only the band at 693  $m\mu$  due to vitamin  $A_2$  appears in this spectrum. These alewives, anadromous teleosts, accompanied the lampreys used in the present experiments in their spawning migration.

FIG. 4. Spectrum of the antimony chloride reaction with a chloroform extract of the livers of late larvae of *P. marinus*. The spectrum shows the presence of the vitamin  $A_1$  band at 618  $m\mu$  alone.

in their spawning migration by a large number of alewives, anadromous teleosts coming in from the sea for the same purpose. The retinas and attached pigment epithelia of a number of these fish were treated precisely like the lamprey tissues. They yielded the spectrum shown in Fig. 3, a single band at 693  $m\mu$  due to vitamin  $A_2$ . Thus the retinal vitamin A patterns of the lamprey and alewife run parallel with their salinity relations in spite of their wide phylogenetic separation.

Compared with freshwater and anadromous teleosts, however, the lamprey retina contains an extraordinarily small amount of vitamin  $A_2$ . If its content is stated as 1, that of the pickerel retina is about 5, the calico bass 14, the white perch 25, the alewife 65, and the carp 200 (Wald, 1938-39).<sup>2</sup>

For this reason porphyropsin must appear in very low concentration in the lamprey retina, so low as to discourage an attempt on our part to extract it. But both the presence of porphyropsin and its low concentration can be inferred from earlier observations. Kühne (1878 *a, b*) reported that retinas of dark adapted *P. fluviatilis* display a strikingly feeble color, which despite its low saturation could be identified clearly as purple, inclining in hue toward violet or bluish. Walls (1935) describes the color of dark adapted *P. marinus* retinas as "truly purple (rhodopsin is usually red in color)." The purple color is characteristic of porphyropsin.

#### Liver

The larval lamprey possesses a liver, gall bladder, and bile duct of normal appearance. During metamorphosis both the latter structures are lost, and with them all direct connection with the intestinal lumen. Yet the liver grows thereafter to a large size, and presumably retains important metabolic functions. In the migrating adult it is deep green in color, due apparently to suffusion with bile pigments.

Fresh livers of late larvae were extracted by shaking exhaustively with chloroform. The extract, tested with antimony chloride, yields the single band at 618  $m\mu$  which characterizes vitamin  $A_1$  (Fig. 4). The fresh liver weighs about 20 mg., and each liver contains about 1.3  $\mu\text{gm.}$  of vitamin A, or about 61  $\mu\text{gm.}$  per gram of fresh tissue. This should be a low but not unusual concentration in the livers of teleost fishes (Sakamoto, 1941).

The identification of vitamin A in adult livers proved to be troublesome.

The tissues were ground fresh with an equal weight of anhydrous sodium sulfate, and were extracted with *n*-pentane in a Soxhlet apparatus. About 10 per cent of the tissue weight of deep brown or greenish brown oil was obtained. A portion of this tested with antimony chloride yielded a highly turbid, impenetrable mixture. The

<sup>2</sup> The very low concentrations of vitamin  $A_2$  in the eye tissues and  $A_1$  in the liver (see below) of the migrating lamprey suggest that it is in a state of severe nutritional deficiency. Probably the lamprey, like a number of teleost fishes, stops feeding toward the beginning of its spawning migration.

oil was saponified in 6 per cent KOH in methanol at 50°C. under nitrogen. The non-saponifiable fraction, extracted with benzine, was surprisingly large, and when tested with antimony chloride yielded brown mixtures with rapidly changing spectra in which the bands of vitamins A could not be identified. Finally a portion of the non-saponifiable oil dissolved in benzine was run through an adsorption column of equal parts magnesium oxide and celite. An orange band was adsorbed high on the column, and below this a broad colorless zone which fluoresced strongly greenish-white in ultraviolet light. Such fluorescence is characteristic of vitamin A<sub>1</sub>. The fluorescent zone was cut away from the rest of the column, eluted with 1 per cent methanol in benzine, and the extract tested with antimony chloride.

The resultant spectrum is shown in Fig. 5. It displays a single band at 612  $\mu$ , due to vitamin A<sub>1</sub>. In the course of this procedure too much material

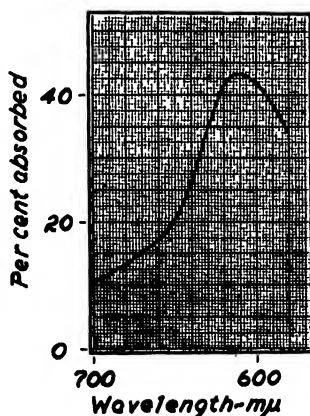


FIG. 5. Spectrum of the antimony chloride reaction with an adsorbed fraction of the non-saponifiable oil from adult lamprey livers. The spectrum shows the presence of vitamin A<sub>1</sub> alone.

was lost to permit an estimate of the vitamin A content of the adult liver, but this was clearly very low.

The lamprey liver therefore reverses the vitamin A pattern of the eye tissues, containing primarily if not exclusively vitamin A<sub>1</sub>. Such reversals of vitamin A configuration in eyes and livers have previously been encountered in freshwater and anadromous teleosts: the carp, chinook salmon, and brook trout (Wald, 1938-39).

#### DISCUSSION

Earlier experiments have demonstrated a genetic correlation between the salinity relations of a wide variety of fishes and the composition of their visual systems. The present observations extend this relation smoothly into the group of cyclostomes. The anadromous sea lamprey possesses a great predominance of the porphyropsin-vitamin A<sub>2</sub> system, precisely like an anadromous teleost.

This observation enormously broadens the evolutionary status of vitamin A<sub>2</sub>, heretofore believed to be associated exclusively with the entry of teleost fishes into fresh water. It shows that the capacity to use and probably to synthesize vitamin A<sub>2</sub> is a deep and recurrent vertebrate property. Since lampreys possess vitamin A<sub>2</sub>, freshwater elasmobranchs may be expected to do so also; and the same may be true even of freshwater invertebrates. In any case the lamprey result calls into immediate question the concept that A<sub>1</sub> is the primitive and fundamental vertebrate vitamin A.

#### SUMMARY

The porphyropsin-vitamin A<sub>2</sub> cycle has been found heretofore only in the retinas of bony fishes capable of existence in fresh water. Cyclostomes, due to their primitive and isolated phylogenetic position, might be expected to possess the rhodopsin-vitamin A<sub>1</sub> cycle common to marine elasmobranchs, almost all marine teleosts, and all terrestrial vertebrates so far examined. Yet the anadromous sea lamprey, *Petromyzon marinus*, possesses primarily the porphyropsin system, like an anadromous teleost. This observation greatly extends the phylogenetic association of vitamin A<sub>2</sub> with the capacity for freshwater existence.

Compared with freshwater and anadromous teleosts, the lamprey retina contains the porphyropsin system in extremely low concentration.

The remaining eye tissues, like the retina, contain both vitamins A<sub>1</sub> and A<sub>2</sub>, the latter greatly predominant. The livers of larval and adult lampreys, however, appear to contain vitamin A<sub>1</sub> alone. This situation also is not without teleost precedent, since the carp and certain anadromous salmonids display similar reversals of vitamin A pattern in the liver and eye tissues.

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# REVIVAL OF MAMMALIAN SPERM AFTER IMMERSION IN LIQUID NITROGEN

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## I

It has been shown (Hoagland, 1933) that we estimate clock or "public" time as longer the higher the internal body temperature. Experimentally we systematically count more subjective seconds to the clock minute the higher the internal temperature and this makes clock time appear to drag. The Arrhenius equation was found to describe the human time sense surprisingly well. The rate of passage of "private" time (counting estimated seconds) yields a  $\mu$  value, or temperature characteristic, of 24,000 calories, a value associated in several studies with cellular respiration.

It is well known that some living organisms can recover after being subjected to the very low temperatures of the liquified gases such as air, hydrogen, and helium. For such organisms at these very low temperatures, with metabolism virtually stopped, time, as a reciprocal rate of some aspect of metabolism, must pass very rapidly as far as the organism is concerned. Since life, and its concomitant aging processes, is a series of chemical events proceeding irreversibly towards death, these temperatures must essentially stop aging processes. Such organisms are, for all intents and purposes, projected forward into the future at these low temperatures. A stay of a century at  $-270^{\circ}\text{C}.$ , the temperature of liquid helium, with subsequent warming and recovery, would, if it were possible to effect, be precisely equivalent to projecting the organism forward a century into the future-- a sort of Wellsian time machine idea.

Luyet, who has made a number of contributions to the field of low temperature effects on organisms, has recently reviewed with Gehenio (1940) the subject of life and death at low temperatures. Some 120 studies, beginning with those of Pictet in 1893, have demonstrated that a variety of small organisms can withstand the temperatures of the liquified gases. These include some bacteria, protozoa, plant cells, and a few metazoans. The procedure in general involves rapid chilling and warming so as to vitrify the organisms and not permit their water to crystallize. Crystallization of water takes time and colloidal solutions with their large particles are relatively slow to crystallize.

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The crystallization range for protoplasmic systems, according to Luyet, extends for only some 30–40° below 0°C. Rapid passage through this range is, for most organisms, essential if they are to survive extremes of chilling. Moreover, the length of time spent at temperatures below the freezing or crystallization range usually, as we should expect, has no effect on the per cent of revivable organisms (Luyet and Gehenio, 1940; Luyet and Hartung, 1941). Organisms of more than a millimeter or so in size cannot survive this treatment since heat cannot be conducted from and to their interiors fast enough on chilling and on warming to prevent internal crystallization and death. The water content of the organisms also is important. In general, organisms with a low water content best withstand vitrification.

*The germ plasm of mammals seemed to us to be of special interest in connection with vitrification since the storage of sperm would be of value in connection with the fertilization to produce offspring perhaps generations after the donor's death.*

Luyet and Hodapp (1938) vitrified and revived an appreciable number of frog sperm after removing some of their water content by plasmolysis with hypertonic sucrose solutions. They were, however, unable to revive any rat sperm. Shettles (1940) in the course of a study of the physiology of human sperm had been able to vitrify and revive a few per cent of seminal human sperm immersed in capillary tubes in the liquified gases and later rapidly warmed.

## II

### *Experiments with Human Sperm*

During the past winter we have confirmed Shettles' findings and extended them. We have been able to revive as many as 67 per cent of sperm after exposure to liquid nitrogen at a temperature of  $-195^{\circ}\text{C}$ . We have used seminal sperm always from the same human donor in a series of experiments attempting to improve the viable yield. Luyet and Gehenio (1940) described immersing small organisms in liquid air by entrapping them in a film on a wire loop 2 to 3 mm. in diameter. In this way there is only a small amount of surrounding liquid and contact is favored with the refrigerant. This method of immersion with certain types of pretreatment of the sperm produced occasional small yields of motile sperm after liquid nitrogen (see Table I). With human sperm our best results came by producing an air emulsion, or foam, of the seminal fluid by bubbling it through a capillary pipette and plunging it, trapped on a very fine wire screen into the liquid nitrogen. The solidified foam is afterwards warmed by quickly plunging it into a small volume of isotonic Locke-Ringer solution, glucose phosphate solution, or blood serum at approximately  $35^{\circ}\text{C}$ . The sperm, motile and dead, are then counted with an hemocytometer. The revived sperm show motility indistinguishable from that of

TABLE I  
*Experiments with Human Sperm*

Date	Method of pretreatment	Method of immersion in liquid nitrogen	Results: Per cent motile after liquid N <sub>2</sub>	Comments
1940				
Nov. 23	None “  Plasmolysis to immobility in 2 M glucose phosphate  Plasmolysis with 1 M glucose phosphate for 2 min.	In film on wire loop Semen in capillary tubes In films on loop  “ “ “ “	Negative “ “  3-18 per cent	Warming solution is isotonic glucose phosphate unless otherwise stated. The warming solution is always tested for its effect on controls  About the same number revivable after this plasmolysis alone and without chilling
Dec. 7	Isotonic glucose phosphate	“ “ “ “	Negative	Dipped in toluol and dry ice mixture at -79° C.
Dec. 8	Various solutions glucose phosphate ranging from isotonic to many times hypertonic	In capillary tubes In films on loop	“ “	
Dec. 13	Very fresh sample no pretreatment Plasmolyse 1 M glucose for 10 min. Plasmolyse 1M glucose for 2 min.	“ “ “ “ “ “ “ “  “ “ “ “  In capillary tube	“ “  0.05 per cent  Negative	Found none alive with < 2' plasmolysed Found none alive with > 3' plasmolysed
Dec. 14	Pretreated as follows; none, plasmolyse with various concentrations of glucose phosphate for from ½ to 40 min.	In film on loop	“	

TABLE I—*Continued*

Date	Method of pretreatment	Method of immersion in liquid nitrogen	Results: Per cent motile after liquid N <sub>2</sub>	Comments
<i>1940</i>				
Dec. 22	Plasmolyse with Ringer $\times$ 5	In film on loop	None	Sperm $\frac{1}{2}$ hr. old
	Plasmolyse with Ringer $\times$ 3.3 For 15 sec.	" " " "	1 per cent	
	Plasmolyse with Ringer $\times$ 3.3 For 5 min.	" " " "	2 per cent	
	Plasmolyse with Ringer $\times$ 3.3 For 13 min.	" " " "	4 per cent	
	Plasmolyse with 1 M glucose phosphate no pretreatment	" " " " Bubbles dropped in	Negative 1 per cent	Note no pretreatment here.
Dec. 25	None	" " "	2 per cent	
	Plasmolyse with 3.3 $\times$ Ringer for 2 to 7 min.	" " "	0.1 per cent	
	None	In film on loop	Negative	
	None	Bubbles and foam	15-30 per cent	Note curious effect of bubble immersion
<i>1941</i>				
Jan. 16	Human sperm in rabbit serum	" " "	20.4 per cent	Controls always taken in conjunction with each test. (See Fig. 1)
	" "	" " "	28.8 per cent	
	None	" " "	12.2 per cent	
	None	" " "	9.2 per cent	
	Semen sperm with isotonic glucose phosphate	" " "	7.0 per cent	
	" "	" " "	3.5 per cent	
	" "	" " "	Negative	
	" "	" " "	1.9 per cent	
	Sperm in rabbit serum again	" " "	12.1 per cent	
Mar. 23	4 min. air drying of smear	Fine mesh copper screen	3.1 per cent	Controls 75 per cent motile
	None	Smear on cellophane	50 per cent	" "
	Sperm in beef serum	Cellophane smear	Negative	Controls 67 per cent motile

TABLE I—*Concluded*

Date	Method of pretreatment	Method of immersion in liquid nitrogen	Results: Per cent motile after liquid N <sub>2</sub>	Comment
1941				
Mar. 23 ( <i>Cont.</i> )	Sperm in beef serum	Bubbles and foam	5.0 per cent	Controls 67 per cent motile
	" "	" " "	15.0 per cent	" "
	None	" " "	20.6 per cent	
	None	" " "	4.0 per cent	
	Sperm in beef serum concentrated × 4	" " "	7.2 per cent	The sperm are all immediately reversibly immobilized by this concentrated serum.
	None	" " "	15.0 per cent	Controls 52 per cent motile
	None	" " "	15.0 per cent	

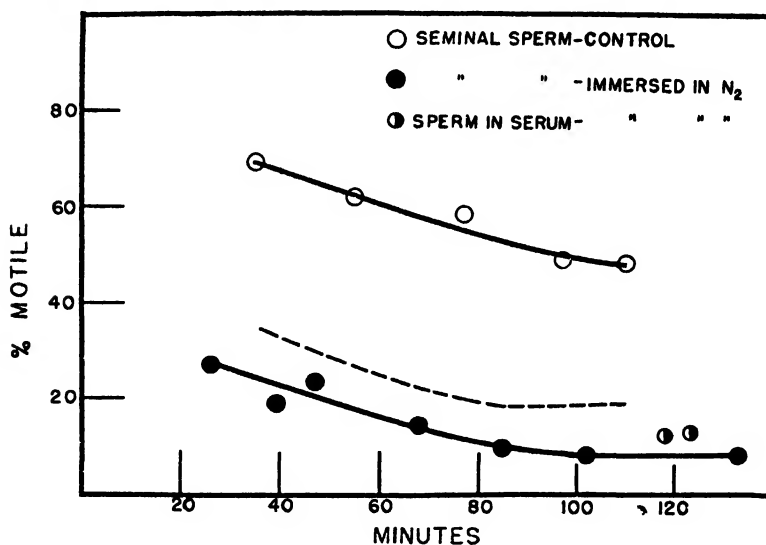


FIG. 1. Upper curve shows effect of age after emission on motility of human seminal sperm. Lower curve shows, for the same sample, per cent motile sperm after vitrification in liquid nitrogen with subsequent warming as a function of age after emission. Middle dashed curve shows the per cent of *living* sperm before vitrification which survived liquid nitrogen and subsequent warming.

normal controls. We have tried a wide variety of plasmolysing solutions as pretreatment to increase the yield. These will be considered later in connection with our experiments with rabbit sperm. In the case of human sperm pretreatment of this kind usually tends to decrease the yield. With the foam

method we typically get yields of 20 to 40 per cent with fresh sperm samples. There is a certain amount of variation from day to day. Table I summarizes our work with human sperm.

Freshness of the samples of human sperm is important. This was also emphasized by Shettles' experiments. Fig. 1 shows a typical curve of per cent recovery of motile sperm after chilling to  $-195^{\circ}\text{C}$ . as a function of the sample's age since emission. The recovery curve parallels a control curve treated the same way except for chilling. Table II shows further data from another sample of sperm indicating the effect of age *in vitro* on the sperms' ability to withstand the temperature of liquid nitrogen.

We do not know how long sperm would remain viable at  $-195^{\circ}\text{C}$ . but we would expect them to do so indefinitely. Shettles kept samples of human sperm at  $-79^{\circ}\text{C}$ . up to 70 days. His data show a small decrease in viability

TABLE II  
*Experiments with Human Sperm*

Age of sperm <i>in vitro</i>	Total motile after liquid nitrogen immersion	Motile sperm in unchilled controls	Living sperm motile after liquid nitrogen immersion
<i>min.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
40	50.0	75	67
65	20.6	—	—
88	—	50	—
130	15.0	52	29
140	15.0	52	29

over this period. But with his small yields and with the wide variability in samples showing these low yields we do not regard this apparent trend in a few experiments as significant.

### III

#### *Animal Sperm*

Human sperm withstand vitrification and revival better than do those from other animals that we have examined. Our preliminary experiments with sperm from rat, mouse, guinea pig, rabbit, and bull yielded completely negative results. We accordingly decided to investigate rabbit sperm systematically. We have studied sperm samples from the vas deferens of 31 rabbits and also 8 samples of ejaculated rabbit sperm. Luyet and Hodapp were unable to revive frog sperm smeared in films on mica and dipped in liquid air without first plasmolysing them in sucrose solutions. After 3 minutes of plasmolysis in 2 M sucrose solution, about 40 per cent of their frog sperm ceased motion but the remainder survived immersion in liquid air if followed by very rapid warming. These revived sperm were motile as long afterwards as were controls.

We have found that this same procedure does not permit any revival of mammalian sperm just mentioned. Human sperm occasionally give by this procedure a small viable yield (Table I). We have plasmolysed rabbit sperm for lengths of time varying from 2 seconds to 30 minutes in many solutions. These include Ringer solution varying from isotonic to ten times isotonic. We have varied the Na, Ca ratio and K, Ca ratios of our pretreating solutions. We have plasmolysed with rabbit, human, and beef serum ranging from isotonic to five times isotonic. We have pretreated the sperm with glycerine, mineral oil, olive oil, citrate, gelatin, petroleum ether, and hypertonic solutions containing butyric acid. All of our experiments were controlled in that the effect on sperm of each pretreating agent was first determined not to produce irreversible immobilization. We have also pretreated rabbit sperm with sterile human seminal fluid.

Following a method of Luyet we have immersed the pretreated sperm samples into the liquid nitrogen by exposing them as films on fine wire loops, by smearing them on mica and cellophane, and by our foaming technique described above in connection with human sperm. We have tried dehydration by the air drying of films and of foam at various rates and have also vacuum dried the sperm at  $-79^{\circ}\text{C}$ . after liquid nitrogen immersion in order to sublime off excess water. In some of the experiments we have used a machine for transferring the sperm into the liquid nitrogen and then retransferring them to the warming medium. This machine effects the transfers in approximately 0.01 second but this does not produce appreciably better results than rapid transfers by hand. All of our experiments have been repeated several times, usually on sperm from different rabbits. Our warming medium has usually been an isotonic glucose phosphate solution experimentally demonstrated to be a good medium for rabbit sperm. Isotonic Locke-Ringer is about equally effective. Rabbit serum maintains motility a little better than either of these media.

Positive results have occurred only as follows: Occasionally we have had approximately 0.1 per cent revival of bull and of rabbit sperm after liquid nitrogen immersion by pretreating them for several minutes with Ringer solution of from 1 to 2 times isotonicity containing double the normal amount of Ca. Very occasionally we have had a comparable yield of motile rabbit sperm after plasmolysing for 5 to 10 minutes in a solution of 5 times the normal Ringer concentration. Our best consistently reproducible results on recovery of motile rabbit sperm after immersion at  $-195^{\circ}\text{C}$ . have been obtained by first drying thin smears of otherwise untreated vas or ejaculated sperm *nearly* to completion on cellophane in air. These, after the chilling, yield from 0.1 per cent to as much as 1.0 per cent motile sperm when rapidly warmed. Pretreatment of rabbit sperm for from 2 to 10 minutes with a solution made up of 0.01 N butyric acid in 3-4 times the normal Ringer or serum concentration also

yields positive and repeatable results to the extent of about 0.1 per cent recoverable motile sperm after liquid nitrogen. All of the plasmolysing solutions that are effective produce complete immobilization on their own account, but this is reversible when the sperm are put in isotonic Ringer solution, serum, or glucose phosphate solution.

#### SUMMARY

1. A wide variety of procedures was used to test the motility of mammalian sperm after plunging them into liquid nitrogen at  $-195^{\circ}\text{C}$ . and later rapidly warming them to  $35^{\circ}\text{C}$ . by plunging them into a suitable balanced and isotonic medium.

2. Using seminal fluid sperm from the same human donor, maximal numbers of motile sperm survived vitrification when the samples were (a) very fresh, (b) untreated with plasmolysing solutions, (c) plunged into the refrigerant in the form of a foam. The maximum yield of motile human sperm recoverable from the liquid nitrogen was 50 per cent. Since in this sample only 75 per cent of the sperm were alive before immersion, 67 per cent of the living sperm survived vitrification.

3. Experiments with sperm from 31 rabbits were made with a variety of conditions of pretreatment to obtain maximal yields of recoverable, motile sperm after vitrification by liquid nitrogen. (a) A consistent recoverable yield of about 0.5 per cent was obtained when the untreated suspension of sperm was smeared on cellophane and *partially* dried in air before immersing in liquid nitrogen. (b) On a few out of many occasions plasmolysis for several minutes with hypertonic Ringer solution gave a recoverable yield of 0.1 per cent as did (c) pretreatment with hypertonic Ringer and butyric acid.

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# THE RADIOACTIVITY OF POTASSIUM FROM HUMAN SOURCES

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The purpose of this paper is to present data which have accumulated in our laboratory over several years tending to show that the relative abundance of the naturally radioactive isotope of potassium  $K^{40}$  in the human body is probably about 2 per cent less than in commercial or "shelf" potassium as supplied in the market. The work was begun on account of the statement of Ernst (1934) based on an inadequate photographic method that  $K^{40}$  is (relative to  $K^{39}$ ) more abundant in living cells. Since the work was begun other papers by Pohlmann (1938), Pohlmann and Netter (1938), Lasnitzki and Oeser (1937), and by A. Lasnitzki (1939) have appeared, all concluding that if  $K^{40}$  is more abundant in biological potassium the difference is not over 5 per cent. Since more data are desirable to improve the precision of the result it seems worth while to publish our data. Moreover, since our experiments were completed, Lasnitzki and Brewer (1941) have investigated the abundance of  $K^{41}$  in potassium from various sources and have found it abnormally abundant in bone (including the marrow) and in plasma but normal in other tissues except tumors where it was low. Presumably similar but smaller differences should be found for the radioactive isotope  $K^{40}$ . In our measurements the  $K^{40}$  was directly determined but the results are not wholly consistent with these expectations.

## *Method*

Human ashes were obtained from the crematorium of the dissecting room. It is probable that some ashes from dogs and occasional pieces of wood and wrappings were included, but all of the ash was biological and most of it was human. The ashes were extracted with hot water. Potassium was precipitated from the filtered extract by ammonium perchlorate. The precipitate was filtered off and washed in water and converted to KCl by heating in the oven. This was changed to  $K_2SO_4$  by dissolving in  $H_2SO_4$  and evaporating to dryness. This in turn was converted to potassium acetate by the addition of the calculated amount of barium acetate. The filtrate was evaporated until it was nearly saturated, the final volume being usually about 10 cc. This solution was sampled with a wash-out pipette for a potassium analysis by a slightly modified Shohl and Bennett method and its radioactivity was measured



by a Geiger-Müller counter described by Bale *et al.* (1939). Other samples of shelf potassium (Baker's analyzed and Kahlbaum's for last 2 samples) were treated in a similar way as controls. The actual number of impulses received by the counting circuit was four times as large as the figures given since only 1 out of 4 impulses was passed on to the counter through the scaling circuit. In some preparations the potassium was dissolved from the ashes in HCl and the calcium was subsequently removed by making the solution alkaline. Excess of barium in preparation No. 7 was removed by hydrogen sulfide and the KCl was recrystallized several times before it was finally prepared as the acetate. In this case a spectrographic analysis of the solution, kindly made for us by Dr. L. T. Steadman, indicated that the concentration of rubidium was not greater than one part in 5000. A trace of barium was present in both samples in apparently equal amounts.

### RESULTS

Nine different comparisons were made between samples of shelf and of human potassium and the results are shown in Table I. Two to six independent chemical analyses for potassium were made on each sample. Lacking sufficient data to calculate a probable error we have quoted only the maximum deviation of these analyses from the average figure which is taken as the concentration of the solution. The solutions were counted in alternation in periods of 15 minutes (usually). The total time devoted to counting each solution is given in the table as well as the total number of counts recorded for each solution and the background count (using distilled water) calculated for the same length of time. The net counts are then divided by the concentration of the solution and by the dilution factor to give the corrected radioactivity in the next to last column. The relative radioactivity of the human K in terms of shelf K as 1.0 is given in the last column. In all cases (but one) the radioactivity of human K is slightly less than that of shelf K and has an average value of 0.98.

#### *Dilution Correction*

In certain of the experiments the potassium concentrations were not the same in the two solutions which were being compared. In such cases it was necessary to make a correction for dilution because the number of counts recorded is not proportional to the concentration but falls off slightly on account of the increasing absorption of the  $\beta$  rays by the solution. To determine the magnitude of this self absorption a strong solution of potassium acetate (80 to 90 gm. per 100 cc.) was diluted to various fractions (0.5–0.8) of its original strength and counts were made in the original and in the diluted solution.

A complete theoretical treatment of the self absorption of  $\beta$  rays is probably too complex to be attempted here but the following approximate formulation will be sufficiently accurate for present purposes. The number of  $\beta$  rays absorbed should be proportional to the number present or to the concentration of  $K^{40}$  and the fraction of those present which are absorbed should be proportional

TABLE I  
*Relative Radioactivity of Shelf Potassium and Human Potassium*

No.	Source of K	Concentration of K acetate	Extreme variation	No. of analyses	Dilution correction	Duration of counting	Total counts	Background counts	Net counts	Counts per unit of K corrected	Relative radioactivity
		<i>per cent</i>				<i>min.</i>					
1	Shelf	94.5	$\pm 1.1$	3	1.0	52	2184	133	2051	21.7	1.0
	Human	71.4	$\pm 0.5$	3	1.04	52	1727	133	1594	21.47	0.989
2	Shelf	94.5	$\pm 1.1$	3	1.0	61	1744	176	1568	16.59	1.0
	Human	66.2	$\pm 0.2$	3	1.05	61	1314	176	1138	16.37	0.987
3	Shelf	93.1	$\pm 0.6$	2	1.0	75	1393	168	1225	13.16	1.0
	Human	59.5	$\pm 0.8$	2	1.06	75	935	168	767	12.16	0.924
4	Shelf	80.7	$\pm 0.6$	5	1.0	60	1675	167	1508	18.69	1.0
	Human	67.2	$\pm 0.9$	6	1.027	60	1448	167	1281	18.56	0.993
5	Shelf	67.2	Diluted from No. 4		1.0	125	3056	353	2703	40.23	1.0
	Human	67.2	Same as No. 4		1.0	125	3004	353	2651	30.45	0.981
6	Shelf	86.8	$\pm 1.0$	8	1.0	140	4275	543	3732	43.00	1.0
	Human	61.1	$\pm 1.6$	8	1.05	140	3328	543	2785	43.41	1.01
7	Shelf	51.0	$\pm 0.2$	3	1.0	50	890	110	780	15.29	1.0
	Human	51.0	$\pm 0.3$	3	1.0	50	870	110	760	14.90	0.974
8	Shelf	32.2	$\pm .02$	3	1.0	200	2414	422	1992	61.86	1.0
	Human	29.6	$\pm .13$	3	1.015	200	2214	422	1792	59.65	0.964
9	Shelf	85.2	$\pm 0.5$	3	1.0	100	2653	200	2453	28.79	1.0
	Human	82.1	$\pm 0.2$	3	1.006	100	2524	200	2324	28.14	0.977

The next to last column is obtained by dividing the net counts by the dilution correction and by the concentration. In No. 8 the densities of the solution were 1.162 and 1.147 whence the concentrations as determined from density tables were 35.8 per cent and 32.5 per cent. Using these values the radioactivity of the human K was 97.7 per cent of that of the shelf K. These solutions were made from No. 7 after the latter had been partially converted to sulfate for analysis. In No. 7 the human potassium had a slightly higher density (1.245) than the shelf potassium (1.235) although the chemical analyses came out exactly equal. The corresponding concentrations are 56 per cent and 53.5 per cent respectively. Using these values the radioactivity of the human K is 93.7 per cent of that of the shelf K. In No. 9 the densities were 1.342 and 1.335 respectively in the shelf and human samples respectively. Using concentrations read from these values the relative radioactivity of the human sample would have been 0.96.

to the density of the solution or to the total concentration of potassium acetate in grams per 100 cc. In other words the number of counts not recorded be-

cause of self absorption should be proportional to the square of the concentration. Consider two concentrations (grams per 100 cc.)  $C_1 < C_0$  giving total counts  $T_1 < T_0$ . Then

$$\frac{T_0 + kC_0^2}{C_0} = \frac{T_1 + kC_1^2}{C_1} \quad (1)$$

This may be transposed to

$$\frac{T_1/C_1}{T_0/C_0} - 1 = \frac{k(C_0 - C_1)}{T_0/C_0} = K \left( 1 - \frac{C_1}{C_0} \right) \quad (2)$$

where the constant  $K = \frac{kC_0^2}{T_0}$  and  $C_0/T_0$  is assumed constant. Now  $T/C$  may be termed the specific radioactivity of the sample of potassium (number of counts per unit of K) so that in words this means that the specific radioactivity of a dilute solution is higher than that of a concentrated solution by a *factor which, for a given value of  $C_0$ , is proportional to the difference in concentration.*

To determine the dilution correction we have made six comparisons between a concentrated solution of K acetate and a solution obtained by diluting that solution to 0.5 or less of its original strength. The results are shown in Table II.

In the first two of these comparisons the dilutions were made by pipettes in the usual way. This, however, introduced some possible error because of the high viscosity of the potassium acetate solution and because of the shrinkage of volume when it is mixed with water. In all the later dilutions the shrinkage of volume was measured and corrected for by measurements of the density of the solutions and in the last three comparisons the dilutions were made altogether on a basis of weight and density rather than by direct volumetric measurement. In Fig. 1 the values of  $\frac{T_1/C_1}{T_0/C_0}$  are plotted against the dilution fraction  $C_1/C_0$ , the results of the last three comparisons being indicated by open circles. With the exception of one point, which seems to be too high all the points lie fairly well along a straight line through the origin of slope =  $K$  according to equation 2. We can find no reason for the high value obtained in this one case; the dilution was checked by density determinations and the solutions were counted with unusual thoroughness on two different counters (3 a and 3 b of Table II) with almost identical results. We have drawn the curve without regard to this aberrant value and have used it for correcting values obtained in Table I. However, if the higher value had been used it would have made the radioactivity of the human potassium still lower in relation to the shelf potassium.

In Table I all the solutions except Nos. 5, 7, and 9 were prepared directly from human ashes as already described. In all these cases some correction for difference in concentration on the basis of Fig. 1 was required. To avoid this

correction Nos. 5 and 7 were prepared from previous solutions of human potassium to have the same concentration as the shelf potassium used for com-

TABLE II  
Data for Dilution Correction

No.	Concentration of K acetate	Dilution	Density	Duration of counting	Total counts	Back-ground counts	Net counts	Counts per unit of K corrected	Relative radio-activity
	<i>per cent</i>			<i>min.</i>					
1	94.5	0.7		40	1141	120	1021	10.8	1.0
	66.2			40	874	120	754	11.4	1.054
2	101.0	0.6		70	1531	145	1386	13.82	1.0
	60.6			70	1040	145	895	14.77	1.069
3a	78.0	0.715	1.328	90	2585	332	2253	28.9	1.0
	55.8		1.245	90	2072	332	1740	31.2	1.080
3b	78.0	0.715	1.328	90	2374	215	2159	27.7	1.0
	55.8		1.245	90	1877	215	1662	29.8	1.075
4	99.2	0.724	1.395	60	1896	120	1776	17.90	1.0
	71.8		1.302	60	1564	120	1344	18.72	1.045
5	99.2	0.510	1.392	60	1872	132	1740	17.54	1.0
	50.53		1.218	60	1092	132	960	19.00	1.083
6	99.2	0.633	1.395	90	2887	213	2674	26.95	1.0
	62.8		1.267	90	1981	213	1768	28.15	1.044

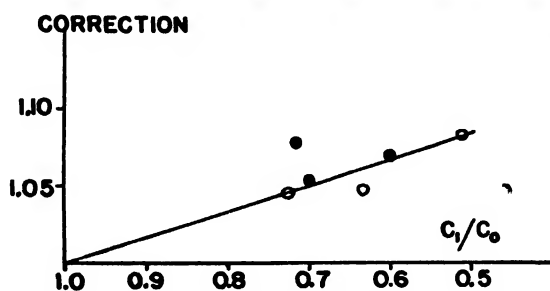


FIG. 1. Correction for dilution of potassium acetate in range where  $C_0$  = about 90 gm. of salt in 100 cc. of solution. The correction =  $\frac{T_1/C_1}{T_0/C_0}$  in equation 2.

parison. No. 9 represented a pooled sample of human potassium prepared from all that remained of all the solutions previously used. Before use it was reprecipitated and purified. Since the correction for dilution can be regarded as having only empirical validity and since such a correction introduces many

additional sources of error in the final result most emphasis must be laid upon those comparisons where both solutions were nearly equal in concentration; *i.e.*, Nos. 5, 7, 8, and 9. All these four values agree very closely the average value being 0.975. The average of all the other values where a dilution correction was used was 0.98.

#### DISCUSSION

It is difficult to estimate accurately the reliability of these measurements. The average error of the K analyses, however, is about 2 per cent. Taking all the comparisons together there were 31 analyses of each type of potassium which gives an error of analysis of about 0.36 per cent. The total number of counts recorded for the human K was  $15,092 \times 4$ . Since the random error of counting is proportional to the square root of the number of counts the percentage error becomes 0.41 per cent. The error of a ratio of counts to concentration is then about 0.55 per cent. Taking the average radioactivity of human K as 98 per cent of that of shelf K, it may be stated that a difference of 2 per cent with an error of 0.55 per cent could occur by accident less than once in 1000 times. The error of the final result may also be estimated directly from the results of the nine different comparisons which gave an average value of 0.978 with a probable error of the mean of 0.005. On this basis it may be stated that a difference of 2.2 per cent could occur by chance once in many thousand times. Analysis of our counts on solution 6 for example shows that the ratio of the net counts in the two solutions was determined with a probable error of 0.6 per cent. Thus in any single determination of the relative radioactivity the chief error is in the chemical analyses and no one figure by itself is significantly different from 1.0. Unless, however, there is some consistent error in all the determinations which we have not detected it must be concluded that all nine determinations together indicate a significant deficiency of  $K^{40}$  in human potassium.

This deficiency in radioactivity of human potassium cannot be due to radium salts which are stated to be a common impurity in commercial KCl (Bramley and Brewer, 1938) because all of these preparations were sulfated during the procedure. Thus the radium would have been precipitated as sulfate and removed along with the barium sulfate. Spectrographic analysis shows that the rubidium present was too small to influence the count. A separation of isotopes of the magnitude observed is by no means impossible. Even greater separations have been made by distillation of metallic K (Hevesy, Seith, and Pahl, 1931) and by the use of a zeolite tower (Taylor, 1939). In the latter method the heavy potassium exchanges somewhat less readily with other cations combined with the zeolite. It might be mentioned also that the heavy potassium would have a slightly lower diffusion rate. Taking the diffusion rates of the two isotopes as inversely proportional to their atomic weights, their ratio would be  $\sqrt{39}/\sqrt{40} = 0.987$  which is not far from the difference observed.

In most cases the shelf potassium used for comparison was reprecipitated, etc. in the same way as the human potassium so that the presence of radium emanation which is reported in commercial potassium<sup>1</sup> could not have vitiated the results. In a few cases, however, the commercial K acetate was used as purchased. To test for radium emanation in such a solution we have compared a boiled (15 minutes) and an unboiled sample of shelf K acetate and have found no change due to the boiling except a possible slight (1 per cent) *increase* in radioactivity without appreciable (0.2 per cent decrease) change in concentration as shown by density.

Assuming that the human potassium does contain only about 98 per cent of the normal radioactivity found in shelf potassium the reasons for this difference may now be considered. One suggestion is that in extracting potassium from the human ashes the lighter potassium was more completely dissolved, especially from the bones. It seems hardly possible, however, that more than a very small fraction of the total potassium of the soft tissues could have remained behind because of the large amounts of fluid used for extraction. The fraction of the total potassium which could be found in the bones may be estimated at less than 5 per cent of the total. If only 10 per cent remained undissolved in the total ash the radioactivity would have to be 20 per cent greater than normal in this residue to explain the observed difference in the fraction extracted.

A second possibility is that the observed difference indicates that the shelf potassium is abnormally high in radioactivity rather than the reverse. We have been unable to obtain information from the manufacturer, however, concerning the origin of the shelf potassium and without this information it is difficult to discuss this point. A comparison between potassium extracted from very old unweathered Canadian granite and commercial potassium has been made by Smythe (1939) but the error of the comparison was of the order of 10 per cent so that the failure to find a difference is of no help in the consideration of our results. Some justification for the use of commercial KCl as a standard may perhaps be derived from the work of Brewer (1939) who used the mass spectrograph to establish the constancy of the  $K^{40}$  isotope in potassium derived from various mineral sources.

One much more probable explanation for our results is that the food which we ingest, coming as it does chiefly from plants, may contain relatively too little of the radioactive isotope (although the reverse seems to be the case in the sap of *Valonia* (Jacques, 1940)). The separation could easily be made by the root hairs which could permit a more ready diffusion of the lighter atoms and could somewhat intensify the difference which would be calculated for water diffusion. Against this suggestion is the finding of Brewer (1937) that certain plants (kelp) have an abnormally great abundance of the other heavy

<sup>1</sup> Personal communication from Dr. Brewer.

isotope  $K^{41}$ . In potato vines, however, he found a slight deficiency of  $K^{41}$  so that at present no general rule seems to hold for plants.

If the plants do not cause the observed deficit in  $K^{40}$  then the separation must be made by the animal body. The intestinal mucosa could hardly be responsible for this effect because it absorbs all but about 2 per cent of the total potassium of the food. It seems more likely that the tubular epithelium of the kidney might be responsible if it reabsorbed the light isotopes more rapidly than the heavy ones. (The glomerular membrane would have the opposite effect, if any.) As the excess of light potassium in the body accumulates, however, the chance of losing this excess in the urine also increases until finally a steady state would be reached in which the isotopic composition of the body potassium would become constant. In this condition the excess of light potassium in the glomerular filtrate would be such that the differential absorbing capacity of the tubules for the lighter atoms would just suffice to give the urinary potassium a normal isotopic composition. At this point the isotopic composition of the body would have become fixed and thereafter the isotopic composition of the urine would necessarily be identical with that of the food. One cannot expect to find, therefore, an excess of  $K^{40}$  in the urine corresponding to its deficit in the body; nor an increasing deficiency of  $K^{40}$  in the urine corresponding to its deficit in the body; nor an increasing deficiency of  $K^{40}$  in the body with increasing age. According to the tubule theory the plasma K should have a low radioactivity similar to the average of the body as a whole, since free exchange of K between plasma and tissues has been established (Noonan *et al.*, 1941). We have, however, some tentative evidence (unpublished) that plasma K is not abnormally deficient in  $K^{40}$  although there is such a deficiency in the K of the erythrocytes. If this is confirmed, the tubule theory must be discarded.

Finally it is possible to suppose that the cell membranes in the body are responsible for a selection of the lighter isotopes. Lasnitzki and Brewer have suggested an ingenious theory which predicts such an effect where the mobilities of the potassium isotopes are considerably diminished by association with constituents of the tissues. Our results seem to be consistent with this theory but it appears that Lasnitzki and Brewer should have found an abnormally low content of heavy isotopes in the tissue instead of the predominantly normal values which they reported. It is not, therefore altogether easy to reconcile our findings with all of those of Lasnitzki and Brewer (1941) although strictly there is no direct factual conflict.

#### SUMMARY

A comparison of the radioactivity of potassium from human and commercial sources indicates that the radioactive isotope  $K^{40}$  is probably 1 or 2 per cent less abundant in human potassium.

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## SOME FACTORS WHICH INFLUENCE THE OXIDATION OF SULFHYDRYL GROUPS

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### INTRODUCTION

The experiments described in this paper show (1) that the oxidation of SH groups by common oxidizing agents such as ferricyanide and Folin's uric acid reagent is inhibited by cyanide and promoted by copper sulfate, (2) that the SH groups of denatured egg albumin can be oxidized by an equivalent amount of ferricyanide even in the absence of denaturing agents, provided aggregation is avoided, (3) that the SH groups of denatured egg albumin are more easily oxidized by some oxidizing agents in urea or guanidine hydrochloride solution than in a solution of long chain alkyl sulfates<sup>1</sup> or in the absence of denaturing agents, and (4) that the SH groups of egg albumin partially hydrolyzed by pepsin are more easily oxidized than the SH groups of denatured but unhydrolyzed egg albumin. It will be shown in another paper that urea and partial hydrolysis promote the oxidation of protein tyrosine and tryptophane groups as well as protein SH groups and that urea promotes the oxidation even of free tyrosine and tryptophane.

Now that it is clear that the oxidation of SH groups even by common oxidizing agents other than oxygen is dependent on cyanide-sensitive catalysts, the possibility must always be considered that a difference in the ease with which two SH compounds are oxidized may be due, in part at least, to a difference in the catalytic impurities present.

All denaturing procedures bring about the same qualitative changes in a protein's properties. The differences in the ease with which the SH groups of denatured egg albumin are oxidized in the presence of different denaturing agents such as alkyl sulfate, urea, and guanidine hydrochloride, however, show

<sup>1</sup> In a previous note (Anson, 1939a) it was stated that urea and guanidine hydrochloride, like Duponol PC, promote the reaction between ferricyanide and the SH groups of denatured egg albumin but that urea and guanidine hydrochloride are less effective than Duponol PC. By this statement was meant merely that the amount of Duponol PC needed to denature egg albumin and thus to make all the SH groups of denatured egg albumin react with dilute, neutral ferricyanide is much less than the amounts of urea or guanidine hydrochloride needed to achieve the same result.

that the exact reactions of a denatured protein depend on what denaturing agent is present. This dependence has also been shown with the nitroprusside test. The SH groups of denatured egg albumin give a strong pink color with nitroprusside in guanidine hydrochloride solution, a weaker color in urea solution, and a still weaker color in alkyl sulfate solution (Anson, 1941).

It is known from earlier experiments that alkyl sulfate, urea, and guanidine hydrochloride all can denature egg albumin, keep the denatured protein in solution, and make its SH groups accessible to titration. These earlier experiments, however, unlike the present experiments, were carried out in such a way that they did not permit any conclusions about differences in the ease of oxidation of the SH groups of denatured egg albumin in the presence of different denaturing agents. In the earlier experiments the SH groups of the denatured egg albumin were oxidized in neutral solution by relatively strong oxidizing agents such as porphyrindin and ferricyanide. Under such favorable conditions for oxidation the SH groups of denatured egg albumin are oxidized in alkyl sulfate solution as well as in urea solution. They are oxidized, as will be shown, even in the complete absence of substances such as urea and alkyl sulfate provided the denatured protein is prepared with proper precautions. In the present experiments, however, the conditions for the oxidation of the SH groups of denatured egg albumin are made more unfavorable by making the solution more acid or by using a weaker oxidizing agent, the uric acid reagent. Under the new, more unfavorable conditions oxidation of the denatured protein does not take place in the absence of denaturing agents or even in the presence of long chain alkyl sulfates. When urea or guanidine hydrochloride is added or when the protein is digested then oxidation takes place even under the less favorable conditions, and the effects of urea, guanidine hydrochloride, and hydrolysis on denatured egg albumin are thus readily demonstrated. Similarly the effect of added copper sulfate on the oxidation of cysteine by the uric acid reagent is demonstrated by carrying out the oxidation in an acid solution in which no oxidation takes place in the absence of added copper sulfate. In neutral solution, oxidation takes place without added copper sulfate.

The experiments described in this paper have helped to make clearer the factors which influence the oxidation of protein SH groups and they have suggested experiments with other protein groups. By themselves, however, *the present experiments are not adequate to decide by what structural mechanisms aggregation, denaturing agents, and hydrolysis influence the SH reactions of egg albumin.* Furthermore, although the urea effects which have been observed cannot be imitated by small amounts of copper sulfate, the present experiments do not decide to exactly what extents catalytic impurities contribute to the effects of various reagents on protein SH reactions. Finally, it is not known to what extent the uric acid reagent influences the surface structure and aggregation of denatured egg albumin.

## EXPERIMENTAL RESULTS

*Cyanide.*—The oxidation of the SH groups of cysteine (Mathews and Walker, 1909) and denatured egg albumin (Rosenthal and Voegtlin, 1933) by oxygen is promoted by heavy metal compounds, especially copper salts, and indeed does not take place at all in the absence of heavy metal catalysts (Warburg and Sakuma, 1923). The work on the heavy metal catalysis of oxidation of SH by oxygen has recently been reviewed by Bernheim and Bernheim (1939).

Most commercial samples of guanidine hydrochloride contain impurities which catalyze the oxidation of the SH groups of denatured egg albumin by oxygen. This oxidation can be inhibited by cyanide (Anson, 1941). When the attempt was made to titrate the SH groups of denatured egg albumin in guanidine hydrochloride solution with ferricyanide in the presence of cyanide, added to prevent oxidation by oxygen, it was found that the added cyanide inhibited not only the oxidation of SH by oxygen but also stopped much of the oxidation of SH by ferricyanide. Cyanide also slowed up but did not prevent the oxidation of cysteine by ferricyanide (Anson, 1941). This inhibition of ferricyanide oxidation by cyanide indicated that even the ferricyanide oxidation requires heavy metal catalysts and led to the present experiments with cyanide and copper sulfate.

The oxidations of SH by the uric acid reagent described in this paper are more completely inhibited by cyanide than the ferricyanide oxidations previously studied. 1 drop of 0.1 N K CN in 10 cc. solution inhibits completely the oxidation of cysteine by neutral uric acid reagent. Somewhat more cyanide is needed to inhibit the oxidation of the SH groups of denatured egg albumin in neutral urea solution (Table I).

*Heavy Metals.*—Copper sulfate promotes the oxidation of cysteine by the uric acid reagent and ferricyanide.

The oxidation of cysteine by the uric acid reagent which takes place in alkaline solution (Folin and Looney, 1922) and in neutral solution (Lugg, 1932; Mirsky and Anson, 1935) does not take place at pH 4.8. If copper sulfate is added, however, the oxidation takes place even at pH 4.8 (Table I).

Similarly, in neutral solution 1 cc. of 0.001 M ferricyanide is reduced by 1 cc. of 0.001 M cysteine with disappearance of the brown color of ferricyanide. At pH 4.8 the brown color does not disappear. If copper sulfate is added at pH 4.8, however, the brown color of ferricyanide is replaced by the weak red color of copper ferrocyanide.

How acid the solution of cysteine has to be made to prevent oxidation by the uric acid reagent or ferricyanide varies with different samples of cysteine. Presumably different samples of cysteine contain different amounts of catalytic impurities. The sample of cysteine used for the experiments in Table I was not the most easily oxidized or the least easily oxidized of the samples of cysteine I have encountered. If the reagents were completely free of heavy metal impurities, oxidation would probably not take place even in neutral solution and even a trace of added copper sulfate would promote oxidation.

In comparing the oxidation of cysteine under different conditions it is important to keep the volume of solution in which the reaction takes place constant. At pH 5.2 cysteine is oxidized by the uric acid reagent 15 per cent if the reaction is carried out for 10 minutes in 10 cc. of solution. If, however, the reaction is carried out in 3 cc. with the same absolute amounts of reactants and the solution is diluted to 10 cc. at the end of 10 minutes, then 71 per cent of the cysteine is oxidized. In 6 cc. of reaction solution, the oxidation is 30 per cent complete.

TABLE I  
*Effect of CN and CuSO<sub>4</sub> on Oxidation of SH Groups*

10<sup>-3</sup> mM of cysteine or 10 mg. egg albumin plus 5.9 gm. urea in 10 cc. of 0.1 M phosphate or acetate solution. Oxidation for 10 minutes at 25°C. by 0.5 cc. of uric acid reagent

SH compound	pH	CuSO <sub>4</sub>	KCN	Oxidation
		mM × 10 <sup>3</sup>	mM × 10 <sup>3</sup>	per cent
Cysteine.....	6.6	0	0	100
Cysteine.....	6.6	0	5	0
Albumin.....	6.9	0	0	100
Albumin.....	6.9	0	10	39
Albumin.....	6.9	0	30	10
Cysteine.....	4.8	0	0	0
Cysteine.....	4.8	4	0	88
Cysteine.....	4.8	1	0	46

*Denaturing Agents.*—The SH groups of denatured egg albumin have been estimated in a variety of ways in solutions of alkyl sulfate, urea, and guanidine hydrochloride which serve to denature the protein and to keep the denatured protein in solution. In a neutral solution of guanidine hydrochloride it requires 1 cc. of 0.001 M porphyrindin to abolish the SH groups of 10 mg. of coagulated egg albumin as shown by the abolition of the nitroprusside test (Greenstein, 1938). In a neutral solution of Duponol PC, a mixture of long chain alkyl sulfates, 1 cc. of 0.001 M ferrocyanide is formed when 1 cc. of 0.001 M ferricyanide is added to 10 mg. of denatured egg albumin (Anson, 1939*a*, *b*). The evidence that ferricyanide is oxidizing only SH groups and all the SH groups is that cysteine is the only amino acid known to reduce ferricyanide under the conditions used, that heat denatured egg albumin treated with iodoacetamide in the absence of denaturing agents no longer reduces ferricyanide in Duponol PC solution, that the amount of ferricyanide reduced by albumin denatured by Duponol PC is within wide limits independent of the concentration of ferricyanide, and the pH, time, and temperature of the reaction (Anson, 1939*a*, *b*), that the amount of ferricyanide reduced is the same whether the reaction is carried out in solution of Duponol PC, urea, or guanidine hydrochloride (Anson, 1940, 1941), and that the number of SH groups detected is the same whether one measures the amount of ferricyanide reduced or the amount of ferricyanide, tetrathionate, or *p*-chloromercuribenzoate needed to abolish the SH groups, as shown by the abolition of the nitroprus-

side test (Anson, 1940, 1941) or the number of SH groups of neutral native egg albumin titrated by iodine in 1 M KI at 0°C. (Anson, unpublished experiments).<sup>2</sup>

Before reagents such as guanidine hydrochloride and alkyl sulfate were introduced, attempts were made to estimate the SH groups of heat coagulated egg albumin which led to SH values that are now known to be about half the correct value. Hopkins (1925) first showed that oxidized glutathione can oxidize some, at least, of the SH groups of denatured egg albumin. When cystine (Mirsky and Anson, 1935) and porphyrindin (Kuhn and Desnuelle, 1938) were added to 10 mg. of coagulated egg albumin, the amount of oxidizing agent reduced was equivalent to only 1 cc. of 0.0005 M cysteine.

The first studies of the reactions between ferricyanide and coagulated SH proteins showed that ferricyanide can oxidize tyrosine and tryptophane groups as well as SH groups and that the amount of ferricyanide reduced is greater the higher the concentration of ferricyanide (Mirsky and Anson, 1936). Later experiments showed that if the concentration of ferricyanide is not too high, ferricyanide reacts only with the SH groups of denatured egg albumin and not with tyrosine, tryptophane, or disulfide groups (Anson, 1939*b*), and that the amount of ferricyanide reduced by SH groups alone depends on the concentration of ferricyanide and on the physical state of the protein, the more the protein is aggregated, the less ferricyanide being reduced (Anson, 1939*a*).<sup>3</sup> Reference was made to earlier viscosity measurements which showed

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<sup>2</sup> There is no significant change in the sedimentation rate of the albumin as a result of the iodine reaction. Thus the oxidation of the SH groups of native egg albumin by an equivalent amount of iodine does not involve polymerization of the protein. The SH groups of egg albumin can be abolished by reaction of the neutral native protein not only with iodine but also with dilute permanganate or with hydrogen peroxide in thousands of times greater concentration than is needed for the oxidation of the SH groups of denatured egg albumin.

<sup>3</sup> When a substance reacts with a protein which is precipitated instead of in solution, the substance has farther to diffuse before it meets a protein particle, it has to penetrate the precipitate, and it reacts with groups whose properties must be changed to some extent by the bonding between molecules in the precipitate. Since a significant fraction of all cellular proteins is not in solution, it would be desirable that the reactions of proteins in the solid state be systematically studied. Some years ago, as a result of some failures in trying to dry crystalline carboxypeptidase without inactivation, I began a study of the denaturation of protein in the solid state. When protein crystals were made insoluble by denaturation procedures the superficial crystalline form was retained (Anson, 1938). The temperature coefficient of denaturation of protein in the solid state was roughly the same as that of denaturation in solution (unpublished experiments). If a solid particle had denatured as a whole, the way a single protein molecule in solution denatures as a whole, the temperature of denaturation would have been almost as sharp as a melting point. When the SH groups of proteins denatured in the solid state were studied by the techniques then available, difficulties were encountered which led to a further study of SH groups. The subject of the SH groups of protein denatured in the solid state has not since been reinvestigated.

that denatured egg albumin can be aggregated even in apparently clear solution and that this aggregation is sensitive to salt (Anson and Mirsky, 1932). Because of the aggregation effect the practical estimation of the SH groups was carried out in the presence of Duponol PC, which acts as a solvent as well as a denaturant. Furthermore, the experiments on the reaction of ferricyanide with the SH groups of denatured egg albumin in the absence of denaturing agents were carried out not with a precipitate but with a solution of denatured egg albumin which reacted much more readily with ferricyanide than the suspensions of coagulated egg albumin previously used. Denaturation was brought about in acid solution and then the denatured protein was cooled and brought into neutral solution. The SH groups of 10 mg. of such a preparation of denatured egg albumin in solution were oxidized 64 per cent by 1 cc. of 0.001 M ferricyanide and oxidized 94 per cent if the ferricyanide concentration was increased 25 times (Anson, 1939*b*). For the present experiments on heat denatured egg albumin in the absence of denaturing agents, denatured egg albumin was prepared in essentially the same way as previously but even more care was taken to avoid precipitation.

The effect of urea on the SH groups of denatured egg albumin can be shown with the uric acid reagent in neutral solution or with ferricyanide in acid solution. The uric acid reagent oxidizes the SH groups of denatured egg albumin in neutral urea solution (Table II, Experiment 1) but not in alkyl sulfate solution (Experiment 2) or in the absence of denaturing agents (Experiment 3). Guanidine hydrochloride gives a precipitate with the uric acid reagent.

Ferricyanide, a stronger oxidizing agent than the uric acid reagent, oxidizes the SH groups of neutral denatured egg albumin even in alkyl sulfate solution<sup>4</sup> (Table II, Experiment 4) or in the absence of denaturing agents (Experiments 5 and 6). In alkyl sulfate solution at pH 4.9, however, ferricyanide oxidizes the SH groups of denatured egg albumin only 21 per cent (Experiment 7). The per cent oxidation is increased from 21 to 86 if urea is present instead of alkyl sulfate at pH 4.9 (Experiment 8). In the absence of denaturing agents denatured egg albumin is insoluble at pH 4.9. At pH 3.9 oxidation by ferricyanide is more complete in guanidine hydrochloride than in urea solution (Experiments 9 and 10).

The blue color formed when the SH groups of a denatured protein are oxidized by the uric acid reagent in neutral urea solution is a convenient measure of the SH content of the protein. The number of SH groups of completely denatured egg albumin, edestin, and tobacco mosaic virus oxidized

<sup>4</sup> The SH groups of denatured tobacco mosaic virus in alkyl sulfate solution are not oxidized even by ferricyanide. Recently Dr. Seymour Cohen has found by measurements of sedimentation rates that tobacco mosaic virus in sodium dodecyl sulfate solution is dissociated into molecules about the size of egg albumin. The failure of ferricyanide to oxidize the virus SH groups in sodium dodecyl solution is therefore not due to aggregation.

in neutral urea solution by the uric acid reagent is the same as the number of SH groups detected by ferricyanide titration in guanidine hydrochloride solution by the procedure described elsewhere (Anson, 1941; Anson and Stanley, 1941). Egg albumin and edestin are rapidly denatured in neutral urea

TABLE II

*Effect of Denaturing Agents on the Oxidation of the SH Groups of 10 Mg. of Denatured Egg Albumin*

Experiment	Denaturant	pH	Oxidant	Oxidation in 10 minutes	Composition of solution				
					Amount of denaturant	Buffer mixture	Amount of buffer	Final volume	Amount of oxidant
				<i>per cent</i>		<i>ratio of 1 M salts</i>	<i>cc.</i>	<i>cc.</i>	
1	Urea	6.6	Uric acid reagent	100	5.9 gm.	1 Na <sub>2</sub> HPO <sub>4</sub> 3 NaH <sub>2</sub> PO <sub>4</sub>	1	10	0.5 cc.
2	Sodium dodecyl sulfate	6.7	" "	0	10 mg.	1 Na <sub>2</sub> HPO <sub>4</sub> 1 NaH <sub>2</sub> PO <sub>4</sub>	1	6	0.5 cc.
3	Heat	6.7	" "	Trace	0	1 Na <sub>2</sub> HPO <sub>4</sub> 1 NaH <sub>2</sub> PO <sub>4</sub>	0.1	6	0.5 cc.
4	NaDS	6.7	Ferricyanide	100	10 mg.	1 Na <sub>2</sub> HPO <sub>4</sub> 1 NaH <sub>2</sub> PO <sub>4</sub>	1	10	50 × 10 <sup>-3</sup> mM
5	Heat	6.7	" "	94	0	1 Na <sub>2</sub> HPO <sub>4</sub> 1 NaH <sub>2</sub> PO <sub>4</sub>	0.1	10	10 <sup>-3</sup> mM
6	Heat	6.7	" "	100	0	1 Na <sub>2</sub> HPO <sub>4</sub> 1 NaH <sub>2</sub> PO <sub>4</sub>	0.1	10	50 × 10 <sup>-3</sup> mM
7	NaDS	4.9	" "	21	10 mg.	1 HAc 2.5 NaAc	0.5	1.6	50 × 10 <sup>-3</sup> mM
8	Urea	4.9	" "	86	1.5 gm.	4 HAc 1 NaAc	0.5	2.5	50 × 10 <sup>-3</sup> mM
9	Urea	3.9	" "	52	1.5 gm.	(2 N HAc)	0.5	2.5	50 × 10 <sup>-3</sup> mM
10	Guanidine HCl	3.9	" "	82	1.5 gm.	4 HAc 1 NaAc	0.5	2.5	50 × 10 <sup>-3</sup> mM

solution in the presence of the uric acid reagent. Tobacco mosaic virus, however, is not rapidly denatured by neutral urea and the native virus does not reduce the uric acid reagent in urea solution. The virus, therefore, is denatured in acid urea solution before the estimation of the SH by the uric acid reagent in neutral solution.

Greenstein (1938, 1939) titrating with porphyrindin, a much stronger oxidizing agent than the uric acid reagent, found that fewer SH groups of egg albumin, edestin,



and globin were made titratable by urea than by guanidine hydrochloride. In the light of the present experiments it is unlikely that, as Greenstein supposed, urea failed to "liberate" all the SH groups of his proteins. Denatured SH proteins give a much weaker pink color with nitroprusside in urea than in guanidine hydrochloride solution (Anson, 1941). It is therefore possible that a negative nitroprusside test is not a suitable end-point for the porphyrindin titration in urea solution.

Urea makes the pH of buffers more alkaline. This effect is especially marked with acetate buffers. When a comparison is made between an oxidation in the presence and absence of urea, therefore, a more acid buffer is used in the presence than in the absence of urea so that the pH, as measured by the glass electrode, is the same in both cases (Table II, Experiments 7 and 8).

When urea or guanidine hydrochloride is used a considerable part of the total volume of solution is occupied by the denaturing agent. Thus for a given total volume of solution the concentration of reactants per gram of water is greater in the presence than in the absence of urea and guanidine hydrochloride. An increase in concentration of reactants per gram of water, however, favors oxidation, as has been pointed out. Thus when a comparison is made of a particular oxidation in the presence and absence of urea and guanidine hydrochloride, the reaction is carried out at constant concentrations of reactants per gram of water not per cubic centimeter of solution. The exact amount of water in a saturated urea solution which is available for a protein reaction is not known.

The uric acid reagent not only does not oxidize the SH of denatured egg albumin in neutral alkyl sulfate solution itself but interferes with the oxidation by ferricyanide in alkyl sulfate solution. Tungstate does not interfere with the ferricyanide oxidation. Both the uric acid reagent and tungstic acid, however, slow up the reaction between ferrocyanide and ferric sulfate to form Prussian blue. No study was made of the mechanisms of these inhibitions.

Alkyl sulfates inhibit the oxidation of the SH groups of denatured egg albumin in urea solution by the uric acid reagent. This inhibition is greater the higher the concentration of alkyl sulfate, and it is greater with Duponol PC, a mixture of long chain alkyl sulfates, than with pure sodium dodecyl sulfate. Thus detergents may act as inhibitors as well as solvents.

*Hydrolysis.*—The effect of hydrolysis in activating the SH groups of denatured egg albumin can be demonstrated with either the uric acid reagent or with ferricyanide. Denatured egg albumin is not oxidized by the uric acid reagent in neutral solution in the presence or in the absence of alkyl sulfate (Table II, Experiments 2 and 3). In contrast, all the SH groups of a peptic hydrolysate of egg albumin are oxidized by the uric acid reagent in neutral solution either in the presence or in the absence of alkyl sulfate (Table III).

At pH 4.8 in a solution of sodium dodecyl sulfate, 1 cc. even of 0.05 M ferricyanide oxidizes 10 mg. of egg albumin only 15 per cent. Under the same conditions the hydrolysate is oxidized 83 per cent (Table III).

78 per cent of the SH in a peptic digest of egg albumin is precipitated by the uric acid reagent, which is a phospho-18-tungstic acid (Wu, 1920), or by saturated ammonium sulfate. The SH which is precipitated is either a part of peptides precipitable by phospho-18-tungstic acid (a more satisfactory precipitant for peptides than tungstic acid) or it is adsorbed to the precipitate which is formed. Free cysteine and cysteine added to a peptic digest of egg albumin are not precipitated by phospho-18-tungstic acid. The 22 per cent of the SH of a peptic digest of egg albumin which is not precipitated by phos-

TABLE III  
*Oxidation of SH Groups of 10 Mg. Egg Albumin Digested by Pepsin*  
10 minutes oxidation in 10 cc. of 0.1 M phosphate or acetate solution

Oxidant	Amount of oxidant	pH	Sodium dodecyl sulfate	Oxidation
			mg.	per cent
Uric acid reagent.....	0.5 cc.	6.8	0	100
Uric acid reagent.....	0.5 cc.	6.8	50	99
Ferricyanide.....	$10^{-3}$ mM	6.8	0	99
Ferricyanide.....	$10^{-3}$ mM	6.8	50	100
Ferricyanide.....	$50 \times 10^{-3}$ mM	4.8	10	83

pho-18-tungstic acid is either free cysteine or SH peptides which are not precipitated by phospho-18-tungstic acid.

Pure SH peptides such as would be desirable for studies of the relation of peptide structure to the properties of the peptides' SH groups have as yet not been isolated from enzymatic digests of proteins. The ease with which SH or its oxidation product, S—S, is estimated should facilitate the isolation of such peptides.

If hydrolyzed egg albumin is allowed to stand in neutral solution, the SH groups are oxidized to S—S by the oxygen of the air. As will be described in a later paper, the total S—S present can then be readily estimated by sulfite plus the uric acid reagent.

#### EXPERIMENTAL PROCEDURES

**Reagents.**—Cysteine hydrochloride—Hoffmann-La Roche. Dissolved in 0.1 N HCl, stored at 0°C., and used promptly.

**Urea**—Merck's reagent. The SH groups of denatured egg albumin were stable for one-half hour in the presence of the particular sample of urea used. Other samples contained more catalytic impurities.

Guanidine hydrochloride—prepared from purified guanidine carbonate according to Anson (1941).

Duponol PC—du Pont. Stored as a 10 per cent solution.

Sodium dodecyl sulfate—prepared especially by du Pont through the kind offices of Dr. Samuel Lenher. Not available commercially.

Ferricyanide—purified according to Anson (1941).

Uric acid reagent—prepared according to Folin (1934).

Ferric sulfate with gum ghatti—prepared according to Folin and Malmros (1929).

*Egg Albumin*.—Recrystallized by ammonium sulfate or sodium sulfate to constant SH content, dialyzed, and stored frozen. Once crystallized egg albumin always has a low SH content and sometimes the SH content is not raised to the usual value of 1.2 per cent cysteine even by repeated recrystallizations. When two procedures for the estimation of SH are being compared, the same sample of egg albumin should be used for both procedures.

Egg albumin denatured by heating in acid is prepared as follows. To 6 cc. of 2 per cent egg albumin add 1 cc. of 1 N HCl, place in 50°C. bath for 5 minutes, cool in ice water, add slight excess of 1 N NaOH so that the solution is blue to thymophthalein (about 0.1 cc. 0.1 N NaOH excess per 10 mg. albumin), add water to 12 cc., cool and store in ice water, and use promptly. Egg albumin denatured by trichloroacetic acid and washed by decantation before solution with NaOH gives the same results as egg albumin denatured at 50°C.

To prepare digested egg albumin a pH 2.0 solution containing 10 mg. of egg albumin and 1 mg. of crystalline pepsin per cc. of 0.045 N HCl is kept at 65°C. for 1 hour. The vessel is evacuated and filled with 99.8 per cent nitrogen several times before digestion is begun. If digestion is carried out in air, there is a small loss in SH.<sup>5</sup> The hydrolysate gives no precipitate with hot 0.2 N trichloroacetic acid. Pepsin itself self-digested at pH 2.0 and 65°C. does not give a nitroprusside test in guanidine hydrochloride solution.

The quantities of reagents used, the volume of solution in which the reaction is carried out, the time of reaction, and the percentage oxidation are given in the tables. The pH was measured by the glass electrode.

The reactions with the uric acid reagent are all carried out at 25°C. After the designated time of reaction the solution is diluted to 10 cc., if it already is not 10 cc., and the blue color formed by the reduction of the uric acid reagent is read either against a standard blue solution formed by the oxidation of 1 cc. of 0.001 M cysteine or against a blue glass calibrated with a standard blue solution. When the blue solution contains urea it is necessary in order to have an optically homogeneous solution either to use a dry colorimeter cup or, if the cup contains residual water, to rinse the cup with a urea solution containing 1 gm. of urea per cc. water.

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<sup>5</sup> When egg albumin was hydrolyzed completely by hot, concentrated sulfuric acid in air, only 1 cc. of 0.005 M cysteine was found in the hydrolysate of 10 mg. of egg albumin (Mirsky and Anson, 1935). Half the SH was presumably lost by oxidation and by adsorption to humin. During the partial hydrolysis by pepsin under the conditions described there is no oxidation of SH or formation of humin.

When SH is oxidized by the uric acid reagent, the solutions are prepared and mixed as follows. Into one test tube there are pipetted the uric acid reagent, the 1 cc. of 1 M buffer (except with heat denatured albumin, when 1 cc. of 0.1 M buffer is used), a volume of 0.5 N NaOH equal to the volume of uric acid reagent (this NaOH being added to neutralize the acid reagent), copper sulfate or alkyl sulfate, when they are used, and water to make up a volume 1 cc. less than the final volume desired. 1 cc. of SH solution is pipetted into another test tube. The uric acid reagent solution is poured into the SH solution and the mixed solution poured back and forth. This manner of mixing minimizes formation of turbid solutions and the introduction of catalytic impurities.

When urea is used, it is first placed into a dry test tube to which the uric acid reagent, etc., are then added. The test tube is placed in a 37°C. bath to dissolve the urea and then the solution is brought to 25°C. before being mixed with the SH solution. After the mixing, the mixed solution is centrifuged to hasten the removal of air bubbles.

When both Duponol PC and 5.9 gm. urea are present in 10 cc. solution, some of the Duponol PC comes out of solution in time. Before the colorimetric reading is made, therefore, the solution is warmed to dissolve the Duponol.

When cyanide is added to inhibit oxidation by the uric acid reagent, it is added to the SH solution just before the mixing with the uric acid reagent.

The ferricyanide reactions are all carried out at 37°C. and, except Experiments 7-10 of Table II, are carried out as follows. The reagents and water are mixed in a test tube, the SH solution being added next to last, and the ferricyanide last. 1 cc. of 1 M buffer is used, except with heat denatured egg albumin when 1 cc. of 0.1 M buffer is used. In the experiments with heat denatured egg albumin, furthermore, the ferricyanide is added promptly after the protein. If the heat denatured protein is allowed to stand in the neutral buffer solution, its SH groups become less completely oxidized by dilute ferricyanide. Ferricyanide is added last so that the alkaline heat denatured albumin does not come in contact with the ferricyanide before the protein is neutralized. After the ferricyanide reaction has taken place in 10 cc. for the designated time at 37°C., there are added 0.5 cc. of 2 N H<sub>2</sub>SO<sub>4</sub>, 0.5 cc. of 10 per cent Duponol PC, 0.5 cc. of 0.1 M ferricyanide or 0.5 cc. of water if the solution already contains concentrated ferricyanide, and 0.5 cc. of ferric sulfate. As previously pointed out (Anson, 1939*b*), the Duponol is added to prevent turbidity and the extra ferricyanide to promote the formation of Prussian blue. After 20 minutes the Prussian blue formed is read in red light against either a Prussian blue solution developed from 1 cc. of 0.001 M ferrocyanide or against a blue glass calibrated with a Prussian blue standard.

Experiments 7-10 of Table II are carried out as follows. To a mixture of 0.5 cc. SH solution, 0.5 cc. 1 M buffer, and 0.5 cc. ferricyanide there is added either 0.1 cc. of 10 per cent sodium dodecyl sulfate, 1.5 gm. of urea, or 1.5 gm. of guanidine hydrochloride. The ferrocyanide formed in the alkyl sulfate and urea solutions is estimated as Prussian blue as already described, the solution being diluted to 10 cc. before the addition of ferric sulfate, etc. Guanidine hydrochloride, however, interferes with the complete conversion of ferrocyanide into Prussian blue. So in the experiment with guanidine hydrochloride one measures not the ferrocyanide formed but

the number of SH groups surviving. The protein is precipitated and washed with 0.2 M trichloroacetic acid and the protein's SH groups estimated with ferricyanide in neutral Duponol PC solution.

The experiment on the effect of copper sulfate on the oxidation of cysteine by ferricyanide is carried out as follows. To 1 cc. of 0.001 M cysteine there are added 0.3 cc. of a 1 M buffer containing equal parts acetic acid and sodium acetate, and 0.5 cc. of 0.002 M ferricyanide. The brown color of ferricyanide does not disappear. 5 drops of 0.002 M copper sulfate are added. The brown color disappears immediately and is replaced by the weak red of copper ferrocyanide.

The experiment which shows that 78 per cent of the SH of a peptic digest of egg albumin is precipitated by 0.05 M phospho-18-tungstic acid in 1 N  $\text{H}_2\text{SO}_4$  is carried out as follows. To 2 cc. digest there are added 0.8 cc. uric acid reagent, 5 cc. 2 N  $\text{H}_2\text{SO}_4$ , and water to 10 cc. The precipitate is centrifuged off and its SH content estimated with the uric acid reagent in neutral urea solution. The SH content of the precipitate is the same whether 1 cc. of 0.001 M cysteine is added to the digest or not.

#### SUMMARY

1. Cyanide inhibits the oxidation of the SH groups of cysteine and denatured egg albumin by the uric acid reagent.
2. At pH 4.8 cysteine is oxidized by the uric acid reagent and by ferricyanide in the presence but not in the absence of added copper sulfate.
3. In neutral solution, the uric acid reagent oxidizes the SH groups of denatured egg albumin in the presence of urea but not in the presence of alkyl sulfate or in the absence of denaturing agents.
4. Ferricyanide oxidizes the SH groups of neutral denatured egg albumin even in the presence of alkyl sulfate or, if precautions are taken to avoid aggregation, in the absence of denaturing agents.
5. In acid solution, ferricyanide does not oxidize the SH groups of denatured egg albumin completely. The oxidation is more complete, however, in the presence of urea than in the presence of alkyl sulfate, and more complete in the presence of guanidine hydrochloride than in the presence of urea.
6. The uric acid reagent which does not oxidize the SH groups of neutral denatured but unhydrolyzed egg albumin in the absence of denaturing agents does, under the same conditions, oxidize the SH groups of egg albumin partially hydrolyzed by pepsin.
7. At pH 4.8 in alkyl sulfate solution ferricyanide oxidizes the SH groups of digested egg albumin more completely than the SH groups of denatured but undigested egg albumin.

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# THEORY AND MEASUREMENT OF VISUAL MECHANISMS

## VIII. THE FORM OF THE FLICKER CONTOUR

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### I

The question of the basic analytical form of the flicker recognition curve is of interest in several ways. An acceptable theory of the flicker contour should provide indices of the invariant properties of flicker recognition under different conditions, such as are necessary for the understanding of this kind of visual excitation and for relating its data to other visual measurements. It is also required for the interpretation of the duplex flicker contour as typically found with vertebrates, where two groups of neural effects usually are complexly overlapped in the determination of flicker. The demonstration of the occurrence of the same fundamental form for the flicker curves in each of these two groups independently, in man, is dealt with in the present paper. This substantiates rather directly the procedure already used for the separation of the two groups of effects in duplex flicker contours, and is important for the analysis of their integrative interaction.

An acceptable equation for the flicker contour must first of all describe in a satisfactory quantitative way the interdependence of flash frequency  $F$  and flash intensity  $I$  critical for the response under a given set of conditions, and with reference to known measures and properties of the variances of the data. A given set of conditions involves effective constancy of wavelength composition of light; the form of the light-dark alternation; the light-time fraction in the flash cycle; the temperature of the organism; and certain other variables, such as the position of the light on the retina, the constancy of the pupil opening, and the degree of homogeneity in the state of the tested individuals during the observations. The acceptable formulation must also, however, and as a necessary condition, exhibit under different circumstances properties of its parameters such as are called for by the notion the equation of the descriptive curve is intended to express. In the present case this involves properties of the variation of the measurements as well. In general, testing the properties of parameters is of course much more significant than the indications from simple criteria of curve-fitting. This is especially so for a multivariate situation, in which the exact relationship between any two significant variables depends upon the magnitudes of a number of others.



The visual performance contours of most vertebrates characteristically exhibit the well known duplex form of the curves describing dark adaptation, intensity discrimination, flicker recognition, visual acuity, and the like, as functions of intensity and of time. For the analysis of duplex flicker contours it has been found necessary to extrapolate the high intensity segment toward  $F = 0$ , and to obtain the low intensity contribution by subtraction from the observed course of the data.<sup>1</sup> This procedure depends upon a valid theory of the fundamental nature and form of the flicker contour as it might be observed if there were no overlapping of the two populations of neural effects. It also relies upon the tested interpretation of the resulting properties of the summation (or rather integration) of the two sets of effects in the region where overlapping occurs.

There are two immediate points, namely: (1) the form of the "cone" curve in isolation and (2) the fundamental form of the "rod" curve when not complicated by the overlapping photopic function. The problem is in some respects more interesting when the degree of overlapping of photopic and scotopic effects is typically more complete, as with man,<sup>2</sup> than with certain fishes<sup>3</sup> for which the separation of the two groups of effects is comparatively extreme. The further question as to the mechanism evident in the overlapping of these two groups of effects depends for its resolution upon the satisfactory analysis of these two points. This analysis requires the systematic experimental manipulation of the conditions of visual excitation in such ways as will expose, without overlapping, the forms of the primary "cone" and "rod" curves. The problem of the integration of effects when overlapping does occur is dealt with elsewhere. Throughout we shall for brevity use the labels "rod"- and "cone"-effects respectively for the low intensity and the high intensity segments of the duplex contours. It is not to be presumed that the two segments are taken to describe the intrinsic properties of the retinal rods on the one hand and of retinal cones on the other. We are simply concerned with the fact that two groups of neural effects are involved in the recognition of flicker, and that these two groups have in certain respects demonstrably different properties although integrative relations can occur between them.

## II

It has been urged<sup>4, 5</sup> that the basic form of the uncomplicated flicker contour is given by a probability band in which  $F \pm \sigma_F$  is a normal probability integral

<sup>1</sup> *J. Gen. Physiol.*, 1936-37, **20**, 411; 1937-38, **21**, 17, 203, 315; 1938-39, **22**, 463; 1939-40, **23**, 229, 667, 677; 1940-41, **24**, 505, 635; 1941-42, **25**, 293.

<sup>2</sup> *J. Gen. Physiol.*, 1937-38, **21**, 203; 1940-41, **24**, 505, 635, etc.

<sup>3</sup> *J. Gen. Physiol.*, 1937-38, **21**, 17, 313; 1938-39, **22**, 463.

<sup>4</sup> *Proc. Nat. Acad. Sc.*, 1937, **23**, 71, 516; 1938, **24**, 125, 216.

<sup>5</sup> *Proc. Nat. Acad. Sc.*, 1936, **22**, 412; 1938, **24**, 130; *J. Gen. Physiol.*, 1935-36, **19**, 503; 1937-38, **21**, 17; 1939-40, **23**, 101.

in  $\log(I \pm \sigma_I)$ . Ordinarily,  $F$  is fixed at various levels and critical intensity  $I$  is repeatedly measured; its mean value  $I_m$  is computed;  $F$  is then exhibited as a probability integral in  $\log I_m$ . With repeated flashes,  $1/t$  (where  $t$  is the flash exposure =  $1/F$ ) is a measure of the capacity to distinguish separateness of flashes. But flicker recognition is also governed by the flash intensity  $I$ , and during a finite interval the excitation should be the integral of a frequency distribution of  $-I \cdot d(1/I)$ , and thus of  $d \log I$ .<sup>6</sup> This integral is then equatable to  $1/t$ . The argument for the expected Gaussian form of this integral is the same as with other modes of excitation.<sup>6</sup> Animals possessing but one general kind of retinal receptor cell (cones)—as the turtle *Pseudemys*,<sup>7</sup> the lizard *Phrynosoma*,<sup>8</sup> the zebra finch *Taeniopygia*<sup>9</sup>—give simplex  $F - \log I$  contours under various conditions of temperature, light-time fraction, retinal area, and wave-length of light, which are well described by the probability summation. It also applies for an animal possessing only retinal rods,<sup>10</sup> and for invertebrates having uncomplicated simplex performance contours.<sup>11</sup> With arthropods the complication usually due to the gross curvature of the optic surfaces<sup>12</sup> has been satisfactorily accounted for.

The three parameters of the probability summation— $F_{max}$ ; abscissa of inflection  $\tau'$ ; standard deviation  $\sigma'_{\log I}$  of  $dF/d \log I$ —while specific for the kind of animal are found to exhibit consistent, non-specific, kinds of relations to temperature, number of retinal units involved, light-time fraction, and wave-length of light. In forms with which adequate cross-breeding experiments can be made these parameters are also found to demonstrate their character as natural invariants.<sup>13</sup>

It will be shown here that when the  $F - \log I$  contour (man) is determined entirely within the normal fovea, where by the usual tests cones alone are presumed to occur, it is of simplex form adhering well to the probability summation over the whole testable range. It is also shown that by novel extensions and developments of the procedure for flicker observations the rising, low-intensity, "rod" portion of the typical duplex curve can be observed free from "cone" complication, and that this curve then also follows the probability integral form, as already shown for the "rod" contribution as it has been dissected out by subtraction of the extrapolated "cone" curve.

<sup>6</sup> *Proc. Nat. Acad. Sc.*, 1937, **23**, 71; 1940, **26**, 54, 334, 382.

<sup>7</sup> *J. Gen. Physiol.*, 1938-39, **22**, 311; 1939-40, **23**, 531.

<sup>8</sup> *J. Gen. Physiol.*, 1940-41, **24**, 317.

<sup>9</sup> *J. Gen. Physiol.*, 1940-41, **24**, 625; 1941-42, **25**, 293.

<sup>10</sup> *J. Gen. Physiol.*, 1938-39, **22**, 555.

<sup>11</sup> *J. Gen. Physiol.*, 1938-39, **22**, 451.

<sup>12</sup> *J. Gen. Physiol.*, 1937-38, **21**, 223, 463; 1938-39, **22**, 451, 795; 1939-40, **23**, 1; *Biol. Bull.*, 1939, **77**, 126.

<sup>13</sup> *J. Gen. Physiol.*, 1937-38, **21**, 17; *Proc. Nat. Acad. Sc.*, 1937, **23**, 516; 1938, **24**, 221; *J. Gen. Physiol.*, 1938-39, **22**, 463; *Proc. Nat. Acad. Sc.*, 1939, **25**, 171, 176; *J. Gen. Physiol.*, 1939-40, **23**, 143.

The data given graphically in sections III and IV are drawn from series of measurements concerned with the questions of image area, location, wavelength, light-time fraction, and number of illuminated patches as concerned with flicker. These data will be given in full when discussing these questions systematically in subsequent papers. The apparatus and technique have been described already.<sup>14, 15</sup>

### III

Measurements of several types of intensity discrimination have made it clear that with test areas falling entirely within the fovea the performance contours are simplex.<sup>16</sup> These curves are in general accurately described by appropriate forms of the probability summation.<sup>8</sup> The common assumption has been that such contours are simplex because only retinal cones are then involved. The  $F - \log I$  contour in the fovea has been determined on several occasions (white light),<sup>16</sup> but somewhat more elaborate tests are required for the purpose here in view.

Fig. 1 contains measurements from series with one practised observer (W. J. C.), for monocular regard (left eye) under the conditions of instrumentation and procedure described in some preceding papers.<sup>14</sup> We have used an area as large as conveniently possible (a square  $0.6^\circ$  on a side), centered at the fovea by means of an appropriately located very small, red, fixation dot of suitable intensity just above the upper side of the square. In general, less scattered determinations are obtained with colored lights, and for these the scatter of  $\sigma_I$  is less<sup>17</sup> although the average value of  $\sigma_I/I_m$  is very nearly invariant, mean  $\sigma_m$  being of the order of 0.4 per cent throughout; each of the plotted points is the mean of ten measurements. The curves drawn are probability integrals (Fig. 2).

When the  $F - \log I$  contour is determined at several light-time fractions, with conditions otherwise the same, a better test is obtained than by mere replication. This is because the properties of the parameters can then be examined. On the basis of these properties as seen in the analysis of the integrals adjusted to data of this observer from other and larger image areas giving duplex contours,<sup>2, 18</sup> we expect in Fig. 1 to find  $\sigma'_{\log I}$  greater,  $\tau'$  lower,

<sup>14</sup> *J. Gen. Physiol.*, 1938-39, **22**, 341; 1940-41, **24**, 505, 635.

<sup>15</sup> *J. Gen. Physiol.*, 1937-38, **21**, 203; 1939-40, **23**, 531; 1941-42, **25**, in press.

<sup>16</sup> Cf. Hecht, S., and Verrijp, C. D., *J. Gen. Physiol.*, 1933-34, **17**, 251; Hecht, S., and Shlaer, S., *J. Gen. Physiol.*, 1935-36, **19**, 965; Hecht, S., and Smith, E. L., *J. Gen. Physiol.*, 1935-36, **19**, 979; Shlaer, S., *J. Gen. Physiol.*, 1937-38, **21**, 165; Steinhardt, J., *J. Gen. Physiol.*, 1936-37, **20**, 185; and Crozier, W. J., *Proc. Nat. Acad. Sc.*, 1949, **26**, 334, 382.

<sup>17</sup> *J. Gen. Physiol.*, 1941-42, **25**, 89, 293.

<sup>18</sup> Report in preparation.

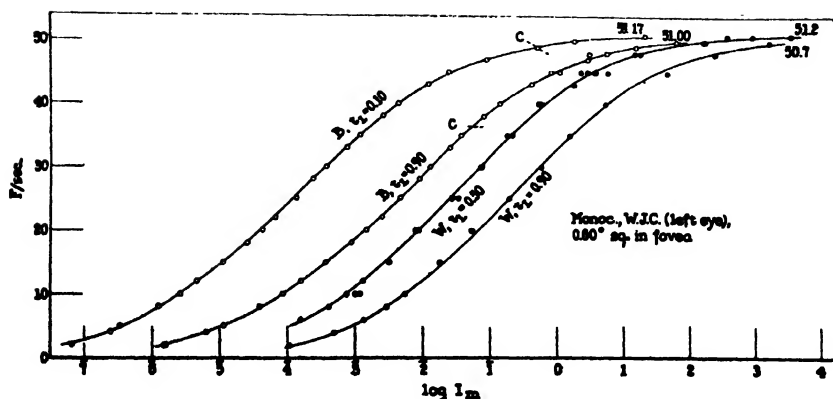


FIG. 1. Flicker response contours for square image  $0.60^\circ$  on a side, within the fovea. To the left, curves for blue (Wratten Filter No. 47),  $t_L = 0.10$  and  $t_L = 0.90$ . To the right, curves for white light with  $t_L = 0.50$  and  $t_L = 0.90$ . The points marked *C* on the *B* curves are the flash intensity levels at which color is just perceptible along the fusion contour. The curves drawn are probability integrals (see Fig. 2), computed to the maximum indicated at the tops of the curves. Each point is the mean of ten determinations. Flash intensities are in millilamberts, blue being matched with white below the color threshold.

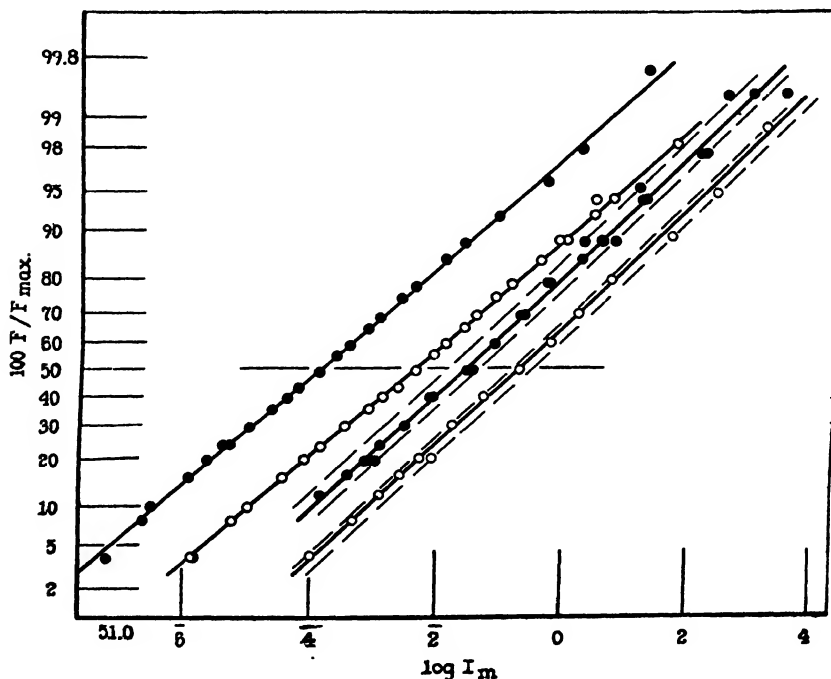


FIG. 2. The data of Fig. 1 displayed on a probability grid.

and  $F_{\max}$ , lower than with larger areas, both for the white ( $W$ ) and for the blue ( $B$ );  $\sigma'_{\log I}$  should be greater and  $\tau'$  should be less for  $B$  than for  $W$ ,  $F_{\max}$ , greater for  $B$  than for  $W$ ; and for each light-time ( $t_L$ ) iraction used  $\sigma'_{\log I}$  should be the same, for  $W$  and for  $B$ . These relations are found. In addition, if the properties of the "cone" segment of duplex contours are of the same kind as those of the exclusively foveal simplex contours, the appearance of color at the fusion point should be, for example, at a rather high flash-intensity separated by  $\log 9$  units on the  $t_L = 0.10$  and  $0.90$  graphs. This is seen in Fig. 1. There are other facts connected with the subjective experiences along these flicker contours, and likewise with the scatter of  $\sigma_{1I}$ , which are consistent with the essential likeness of the properties of these simplex contours and those of the "cone" branches in the duplex cases. We may conclude that the simplex "cone" curve is adequately described over its whole extent by the probability integral.

#### IV

Two methods have been used to obtain directly the practically pure rising "rod" portion of the duplex flicker contour. In one, colored light is used to form a flickered image of suitable area at some distance from the fovea. In the other a flickered field containing several illuminated patches is employed. Qualitatively, the results are of the same kind. The theoretical consequences of the experiments with the second procedure are of considerable interest, but will not be enlarged upon here. One point should, however, be mentioned: under the conditions used it can be shown that the flicker contours due to the non-homogeneous but simultaneous excitation of suitably separated retinal areas are also simple probability integrals, both for the "rod" and for the "cone" segment, and that the rules already found<sup>2</sup> for their integrative overlapping continue to apply. This is manifestly consistent with a central nervous control of the flicker end-point, and clearly with the theory of its statistical determination, rather than with the idea of its government by retinal photochemistry—since the several areas, taken singly, provide quite different contours.

Fig. 3 gives an illustration of results with the first method, using blue light. By comparison with the contours obtained for images closer to the fovea, the form in Fig. 3 is at first sight peculiar. With smaller test areas in the retinal periphery the shape of the contour becomes still more distorted. In the absence of tests with intermediate areas and other retinal locations the extrapolation of the "cone" curves would scarcely be attempted here, but we do have these data. The parameters of the empirically fitted "cone" integrals have the expected interrelations. Their extensions approach  $F = 0$  at just about the intensity where the "rod" curve reaches its maximum (Fig. 3). It is shown that the "rod" data, without subtractive correction for "cone" effects

in this case, are here directly described by the probability integral (Fig. 3). The "rod"  $\sigma'_{\log I}$  is the same for  $t_L = 0.10$  and  $0.75$ ;  $F_{\max.}$  does not perceptibly change, but even for the "cone" branch this change is quite small with colored lights; the shift in  $\tau$  is slightly greater than for the "cone" curves. As for the various cases<sup>2</sup> in which the "cone" curve more or less underlies the whole of the "rod" part, the declining "rod" curve is also given by a probability integral.

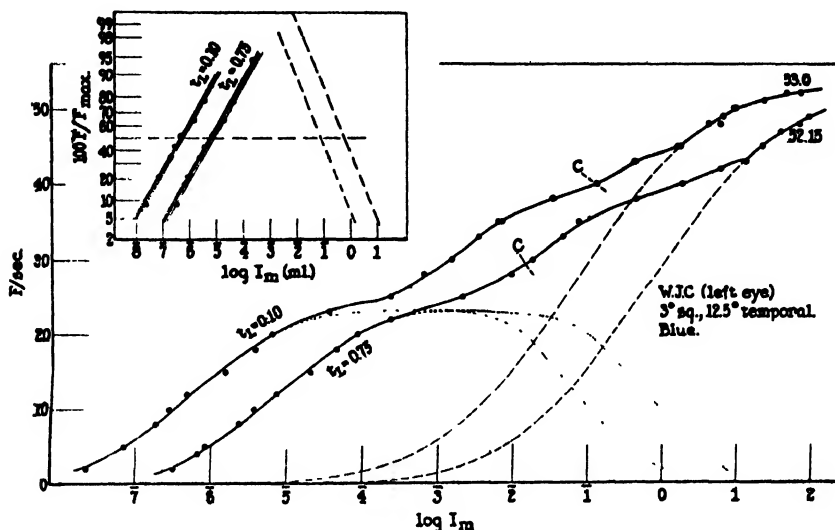


FIG. 3. Flicker response contours for a  $3^\circ$  square centered  $12.5^\circ$  on the temporal side of the fovea, with  $t_L = 0.10$  and  $t_L = 0.75$ . Probability integrals are adjusted to the uppermost short segments, and the "rod" contributions are determined by difference. The inset figure shows that the rising "rod" branches, computed to  $F_{\max.} = 23.3$ , and the declining contributions obtained by ordinate differences, are well described by the probability integral. Flicker contours with structures intermediate between those of Fig. 1 and these of Fig. 3, as well as more extreme instances, are provided by the use of other test areas and retinal locations.

The second procedure used to free the basic "rod" curve from "cone" involvements uses the striped cylinder technique<sup>15</sup> and a subdivided test-field such as is shown in Fig. 4. With high values of  $t_L$  the contours obtained are of the general form seen in Fig. 4. Despite the subdivision of the field the "cone" and "rod" segments are smooth, simple functions which shift systematically when  $t_L$  is changed. The significance of these facts for the theory of visual integration is developed elsewhere. For the present purpose it is sufficient to show that by this procedure the "cone" curve may be made so steep that the rising "rod" branch is almost completely exposed (*cf.* Fig. 4). The extrapola-

tion of the "cone" integrals (Figs. 4 and 5) requires almost no subtraction from the rising "rod" curves, for which the data adhere to the probability summation as shown in Fig. 6.

The probability summation equation applies non-specifically to the relation between  $F$  and  $\log I$  for a great variety of animal types, and for a number of systematically varied conditions. Its three parameters have been experimentally caused to change in such ways as to show that these three independent

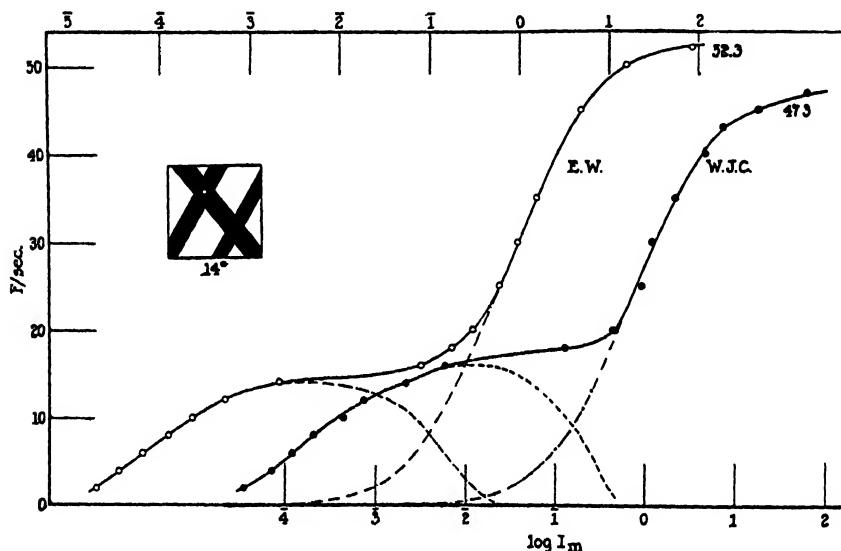


FIG. 4. Monocular flicker contours (left eye, white light) for two observers, employing the method of moving stripes,  $t_L = 0.90$ , and a divided field  $14^\circ$  square with the pattern shown in the inset. The "cone" probability integrals with the indicated maxima are shown in Fig. 5.

parameters are necessary and sufficient. Its derivation is based upon the conception that a large number of neural units are concerned in the flicker response, and that these units fluctuate both in momentary excitability ( $1/I$  for response) and in their capacities to produce elements of neural effect for the determination of the result used as index of response. The analysis of dark adaptation<sup>19</sup> and of intensity discrimination shows that at each instant during the course of progressive changes of excitability a population of neural effects forms a unitary statistical assemblage. This is basically confirmed by the properties of the variation of the critical intensity.<sup>19, 20</sup> It is also strikingly

<sup>19</sup> *Proc. Nat. Acad. Sc.*, 1940, **26**, 334, 382.

<sup>20</sup> *J. Gen. Physiol.*, 1937-38, **21**, 17; 1939-40, **23**, 101; 1940-41, **24**, 505, 635; 1941-42, **25**, 89, 293.

confirmed by the integrated action apparent in our experiments with subdivided fields (Fig. 5, etc.). The statistical basis for the integration of "rod" and "cone" effects along the flicker contour is likewise consistent with this view. As already indicated, the inhibition of some "rod" effects in the zone of overlapping on the duplex curves<sup>21</sup> also follows the simple probability law in all cases examined.

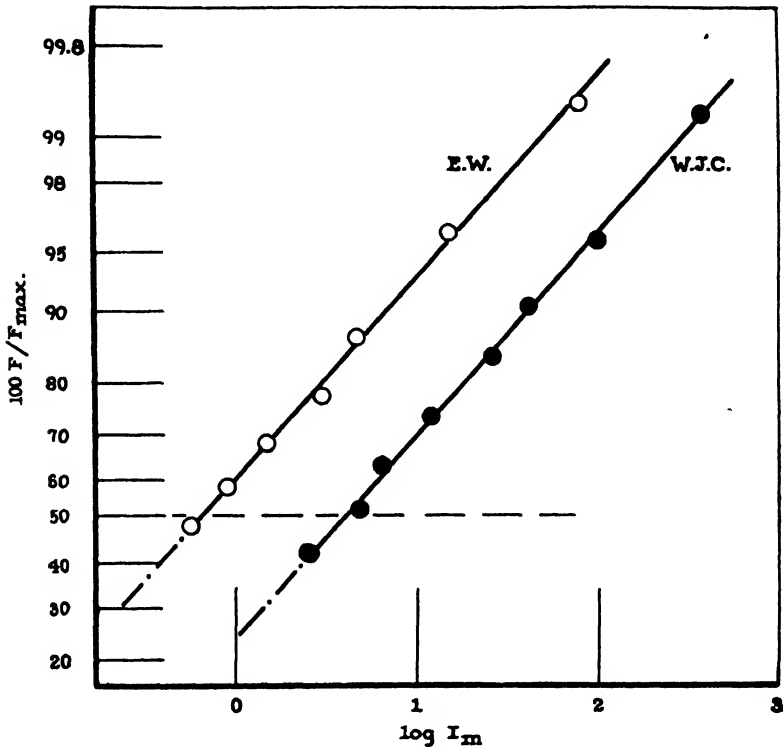


FIG. 5. The upper segments of the curves in Fig. 4 shown on a probability grid.

Since three parameters are required for description of the flicker contour, and since the shape constant ( $\sigma'_{\log I}$ ) can be changed by simply going to another part of the retina or by subdividing the image field, it cannot be held<sup>18</sup> that the photochemical theory of visual data accounts for the form of the curve even under fixed conditions of temperature, light-time fraction, retinal location, and the like. When simplex performance contours are naturally available<sup>7, 9, 11</sup> or are experimentally uncovered, as in the present instances, their forms do not agree with the requirements of photochemical theory any more than do the

<sup>21</sup> *J. Gen. Physiol.*, 1940-41, **24**, 635; 1941-42, **25**, 89, 293, in press.



properties of their parameters;<sup>1, 2</sup> when duplex flicker contours must be dealt with, the transitions from "rod" to "cone" branches are not analyzed at all by the photochemical hypothesis.

In many situations, perhaps in most, which have to be considered by quantitative biological theory it is obvious that many cellular and other structural units are acting at the same time. It has long been recognized that these units

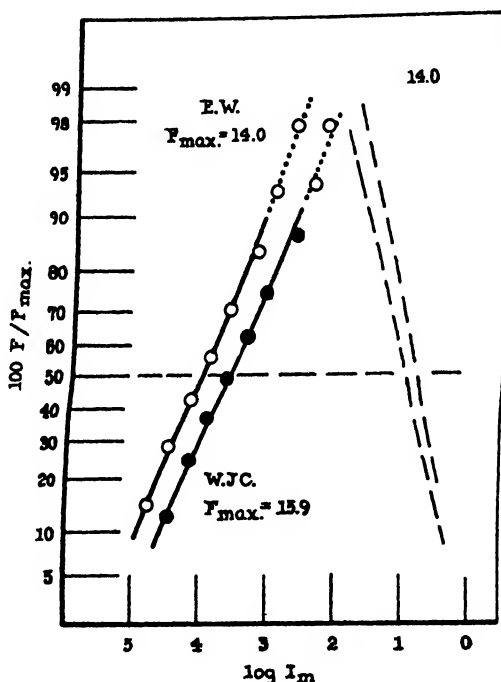


FIG. 6. The "rod" segments of the contours in Fig. 4, on a probability grid. The values of  $F_{\max.}$  are indicated.

are probably never quite identical, and that they spontaneously fluctuate in threshold excitability as well as in performing capacity. There is good reason to believe that the analysis of visual data can illustrate the operation of these factors, under especially favorable circumstances. A primary objective must be the separation of non-specific statistical properties of assemblages of units from those properties which can be used to further the interpretation of intrinsic cellular events, such as those involved for example, in sensory excitation.<sup>22</sup> This procedure will often be confronted by complex interactions and compoundings of the activities of different groups of acting units. Instances, like those

<sup>22</sup> *J. Gen. Physiol.*, 1939-40, **23**, 143; *Proc. Nat. Acad. Sc.*, 1937, **23**, 71; 1939, **25**, 171, 176; 1940, **26**, 334.

discussed in this paper, in which the nature of such integrations can be exposed have therefore a particular interest for the theory of quantitative biology.

## VI

## SUMMARY

Flicker response curves (man) obtained with images formed entirely within the fovea are like those secured with lower animals having only one general class of retinal receptors. They are normal probability integrals ( $F$  vs.  $\log I_m$ ), and the properties of their parameters agree with those for visually simplex animals and for the "cone" portions of contours exhibiting visual duplexity.

By several different procedures, involving experimental modifications of the "cone" curve, the "rod" part of the typical human duplex curve can be obtained free from overlapping by the extrapolated "cone" curve. It then has the probability integral form which the lower segment does not directly exhibit when combined with "cone" effects.

These results are discussed with reference to the statistical nature of the fundamental form of the flicker contour and to the interpretation of duplex curves produced by the neural integration of two independently modifiable groups of sensory effects.



# THE WAVELENGTH SENSITIVITY FUNCTION FOR THE ZEBRA FINCH

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## I

Data on the spectral distribution of visual excitability in birds have been obtained by various procedures, but most frequently by methods deriving from interest in the problems of color vision. Thus spectral lights have been used in tests of intensive discrimination (Lashley, 1916) and of threshold excitability (Watson, 1914, 1915), through use of training procedures (Breed, 1912; Bailey and Riley, 1931; Hamilton and Coleman, 1933; van Eck, 1939) and the pecking selection of colored objects (Hess, 1907, 1908; Katz and Revesz, 1909; Henning, 1920; Honigmann, 1921; *cf.* also Beuner, 1938) or of objects on a colored field (van Eck, 1939) in lights of different colors. For reasonably full measurement of visual excitabilities these methods are deficient. Disturbances of breathing rhythm (Rouse, 1905), the electrical responses of the eye to excitation by light (Piper, 1905; Kohlrausch and Brossa, 1914; Kohlrausch, 1918; *cf.* especially Kemp and Graham, 1935, and Graham, Kemp, and Riggs, 1935), and the contraction of the iris (Hess, 1910; Laurens, 1923; Hecht and Pirenne, 1940-41) variously have been employed for objective determinations of the relations between wavelength and excitability, and to some extent the dependence of this upon intensity. These procedures all have had certain serious defects.

The flicker contours for certain birds, as determined from the observed occurrence of nystagmus, are situated at comparatively low levels of flash intensity (Crozier and Wolf, 1940-41 *a*). This makes it possible to examine the dependence of the flicker contour upon wavelength composition, although not with lights strictly monochromatic, by using flash cycles with small percentage light-time (*cf.* Crozier and Wolf, 1940-41 *b*, etc.). The diurnal bird here employed, the Australian zebra finch *Taeniopygia castanotis* (Gould), histologically has but one general class of retinal photoreceptors, which are cones by the customary criteria. Under various conditions of excitation it is found to provide a flicker contour which is simplex, a normal probability integral (*cf.* Crozier and Wolf, 1940-41 *a*, 1941-42 *d*, etc.). The changes in this contour might thus be conceived to reflect properties of cone excitability, as expressed in behavior.

While the experiments provide no direct evidence regarding "color" vision, it will be pointed out that certain comparisons and deductions are possible on the basis of measurements by the same method with other organisms. The general advantage of the flicker method is that it permits use of the same technique with different kinds of animals, over a large range of intensities. The nature of the flicker contour is moreover now well enough understood (*cf.* Crozier and Wolf, 1941-42 *a*) to make interpretations possible in terms of the properties of its parameters, and thus to be based upon invariant indices of excitability such as are independent of the intensity level. Some of the considerations which enter have been referred to in a preceding paper (Crozier and Wolf, 1941-42 *b*). The question is strictly one of flicker excitation in relation to wavelength, since even in man the occurrence of subjective color has no effect upon the form or properties of the flicker contour as such (Crozier and Wolf, 1941-42 *b*).

## II

Zebra finches from the stock used in our experiments with white light (Crozier and Wolf, 1940-41 *a*) were tested in the manner already described. Four individual birds, males, were involved throughout. No differences of serious degree were found among them. Tests were made with violet, blue, green, yellow, and red filtered from the white. These changes in wavelength composition were produced respectively by filters Corning 511, Wratten 47, 58, Schott GG-11 and Wratten 70. These filters (excepting the yellow) we have also used for observations with man (Crozier and Wolf, 1941-42 *b*). Save for the red, practically complete contours were obtainable by working with a flash cycle having  $t_L = 0.10$ . A sufficient number of measurements were also made at  $t_L = 0.50$  to show that the same order and relative spacing of the contours is found as with  $t_L = 0.10$ .

The data are given in Table I. The variation indices (Fig. 1) follow exactly the rule (as to the mean values *and* the scatter of  $P.E._1/I_m$ ) already found for  $t_L = 0.10$  and  $0.50$  with white light. The intensity units are given in terms of millilamberts for *white* light, the figures for the selected spectral regions being equated to the white by means of thermopile measurements (Mohl large surface vacuum thermopile and sensitive galvanometer used as a null instrument, with rock salt filter). The data for white light,  $t_L = 0.10$ , are taken from another report;  $\sigma'_{\log I}$  is the same as for  $t_L = 0.50$  (Crozier and Wolf, 1940-41 *a*).

In Figs. 2 and 3 it is apparent that there occur certain slight but definitely systematic departures from the curves drawn, essentially reproduced in the several contours. Analogous departures have been noted and accounted for in our data on the turtle *Pseudemys* (Crozier, Wolf, and Zerrahn-Wolf, 1938-39). There it was shown that the small but real differences between

individuals did not wholly account for the deviations, but that they were correlated with the use of particular decimal filters for the control of intensity. The same correlation is apparent in the present measurements. The filters were calibrated with care, but defects in calibration as well as the fact that the filters are not exactly "neutral" seem to be responsible. It is not unimportant that this kind of evidence as to the precision of the observational methods should be automatically given by the measurements.

TABLE I

*Data for the Flicker Response Contours with Violet, Blue, Green, Yellow, and Red Lights*  
(See Text), with the Zebra Finch;  $t_L = 0.10$

The intensities are in terms of (*white*) millilamberts, the scales for each colored light being adjusted to that for *white* by means of thermopile measurements (excluding infrared). Each mean critical flash intensity  $I_m$  is the average of three measurements with each of four birds, the same individual birds being used throughout. The P.E.'s are for the dispersions of the four individual averages.

<i>F</i>	Violet		Blue		Green		Yellow		Red	
	$\log I_m$	$\log P.E._{1f}$	$\log I_m$	$\log P.E._{1f}$	$\log I_m$	$\log P.E._{1f}$	$\log I_m$	$\log P.E._{1f}$	$\log I_m$	$\log P.E._{1f}$
<i>per sec.</i>										
5	7.8343	9.7160	6.5417	7.0398	6.0582	8.4302	6.6673	8.8280	4.5628	5.0964
10	6.5331	8.7602	5.2561	7.6836	6.7998	8.7312	5.3725	7.4432	3.2980	5.5948
15	5.0658	7.4948	5.7771	6.2523	5.3146	7.2083	5.8945	7.7881	3.8109	4.1504
20	5.5587	6.0062	4.2811	5.1069	5.8132	7.9775	4.3846	5.0562	2.3438	4.3750
25	5.9860	6.5857	4.7101	5.0593	4.2207	6.5647	4.7923	6.8631	2.7062	4.9868
30	4.3070	6.7249	4.9926	5.4726	4.5301	6.7849	3.1205	5.1028	2.9843	3.3513
35	4.6330	6.9969	3.3760	5.8359	4.8991	5.1514	3.4914	5.8077	1.3568	3.2134
40	3.0272	6.9916	3.7146	5.7474	3.2481	5.6967	3.8509	4.1449	1.7053	3.9162
45	3.5107	5.9588	2.1946	4.6755	3.7091	4.0385	2.2974	4.2302	0.2039	2.3847
48									0.5818	2.8191
50	2.1820	4.6728	2.8923	3.2953	2.5142	4.9459	2.9993	3.0718		
52	2.7040	3.2242	1.3437	3.4907			1.4261	3.6182		
53					1.2796	3.7757				
54	1.5324	3.8476	0.1394	2.2112			0.2342	2.4293		
55					0.3408	2.7357				

## III

The measurements of  $\log I_m$  for the filtered lights are exhibited as a function of  $F$  in Fig. 2. The curves drawn are normal probability integrals (Fig. 3). The slope constants ( $\sigma'_{\log I}$ ) for these curves are not certainly different, within limits of error. Even for man the corresponding differences are slight. They are all a little, but perhaps not significantly, different from that of the curve for *white* with  $t_L = 0.50$  as already published ( $t_L = 0.50$ , Crozier and Wolf, 1940-41 a); for the latter, and for the present  $t_L = 0.10$  series with which its slope agrees,  $\sigma'_{\log I}$  is a little greater.

In terms of the indications drawn from our studies of human (cone) visual

excitation, and of cone excitation in lower vertebrates, this means that the *number* of cone units involved, whether the stimulating light is V, B, G, Y, R, or W, is pretty much the same. These indications obtain for sundry types of intensive discrimination (Crozier, 1940 *a, b*) in addition to flicker

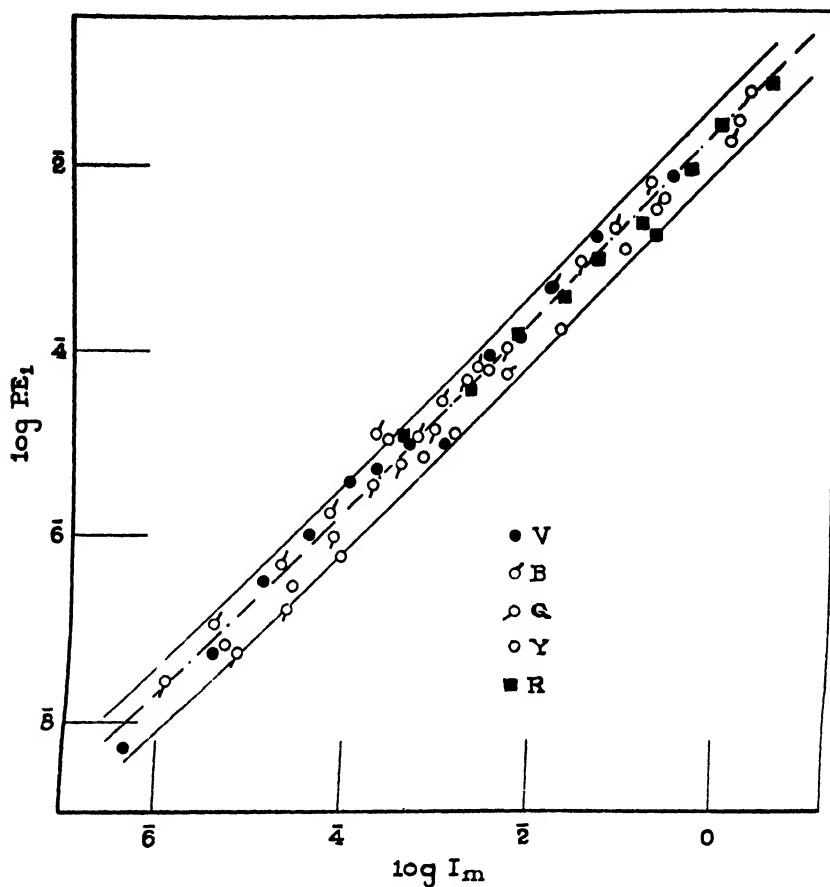


FIG. 1. Variation of critical intensity as a function of flash frequency and spectral region. The band has a slope of 1. Data in Table I; see text.

(*cf.* Crozier and Wolf, 1939-40, 1940-41 *a*, 1941-42 *b, c*, etc.). Only for the red (R) can we be reasonably sure that the asymptotic maximum  $F$  is actually a little different from that for the others (*cf.* Figs. 1 and 2). Thus from the standpoint of (1) numbers of units excitable and (2) the numbers of elements of effect obtainable, as regards flicker recognition, the differences among the influences of the several spectral regions is very slight indeed. It is again illustrated, however, that light filtered from a white can produce a higher

$F_{\max.}$  than that observed with the unfiltered white (*cf.* Crozier and Wolf, 1941-42 *b*). With this bird, the *red* exhibits this effect, whereas with man it is found in the *violet* and *blue*.

In another general respect there is an interesting parallelism with the corresponding curves for man (Crozier and Wolf, 1941-42 *b*). Either from the energy standpoint, or from the standpoint of visual photometry (man), curves for lights from the blue end of the spectrum lie below that for white, while the

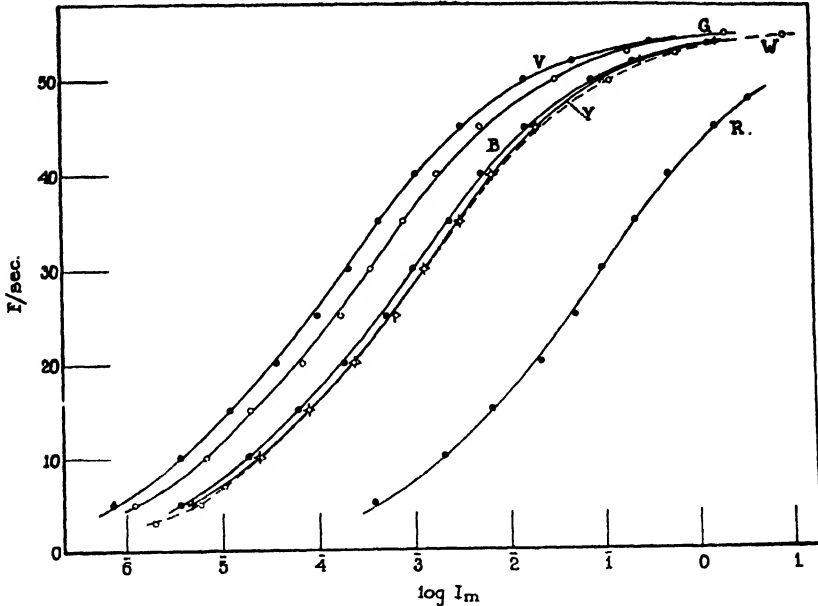


FIG. 2. Mean critical intensities for response to flicker by the zebra finch, with different spectral regions and white. Data in Table I; see text. The intensity scale is in millilamberts for *white* light, the intensities for the colored lights being adjusted to this on the basis of thermopile measurements; the scale is thus a relative energy scale. (The minor systematic deviations from the probability integrals drawn are discussed in the text.)

curve for red may fall at intensities higher than the curve for white. In the human case, with retinal areas of moderate size,  $F_{\max.}$  at a given  $t_L$  is more decidedly a function of wavelength, so that adequate comparisons required finding for each color the value of  $t_L$  serving to bring the curve to the same  $F_{\max.}$  The mean energy flux for the inflection point on the ("cone") curve ( $\tau'$ ) could then be computed. The comparison of these quantities showed that excitation by the white was approximately an average of that due to primary parts of the spectrum, and certainly not in any sense a simple sum of primary excitations. (Even without correction to the same  $F_{\max.}$  this conclusion is qualitatively correct.)



For the zebra finch this comparison can be made a little more directly. Thus for  $t_L = 0.10$  the maxima for V, B, G, and Y are practically the same. That for R could not be reduced to the same level by changing  $t_L$ . For the white,  $F_{\max.} = 55.2$  at *ca.*  $t_L = 0.50$ , with  $\tau' = \text{ca } 3.8$ , as we know from other work. We also know from these additional observations that  $\sigma'_{\log I}$  is independent of  $t_L$  within these limits (*cf.* Fig. 2, and Crozier and Wolf, 1940-41 *a*). We can then compare the values of the mean relative energy flux for 50 per cent activation of all the elements potentially excitable by white and by the

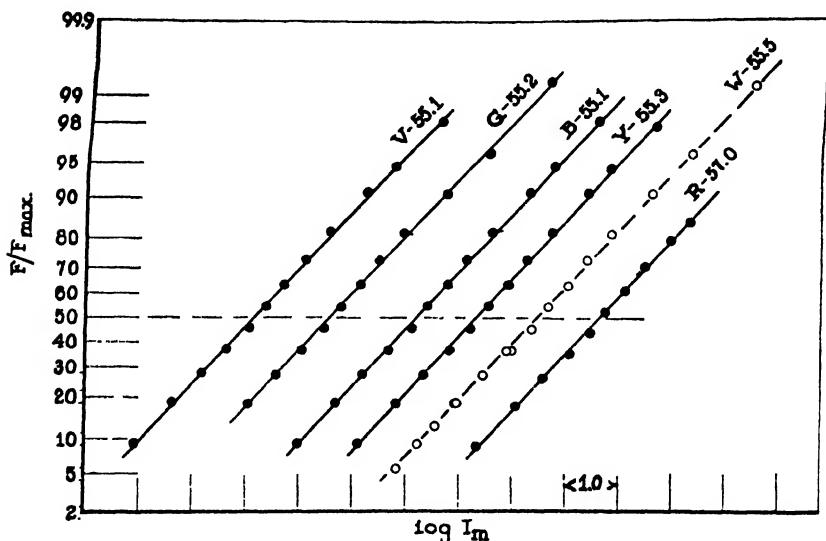


FIG. 3. The data of Fig. 2 shown on a probability grid. The lateral separation of the curves is arbitrary, for convenience. The slopes for the different contours differ only very slightly; that for the red (R) is a little lower than for the others, but in the case of the white (W) the slope is lower still. The maxima (asymptotic  $F_{\max.}$ ) to which the curves have been computed are indicated.

different colors, although the properties of the R curve compel the comparison to remain qualitative.

The relative mean energy fluxes at the inflection points of the curves in Fig. 2 are ( $t_L = 0.10$ ):

$$\begin{aligned} V &- \bar{5}.063 \\ B &- \bar{5}.971 \\ G &- \bar{5}.334 \\ Y &- \bar{5}.492 \\ R &- \bar{3}.867 \\ (W &- \bar{5}.950), \end{aligned}$$

and for W, at about the same  $F_{\max.}$ ,  $t_L = 0.50$ , the number is  $\bar{4}.498$ . Flicker excitation by the white is clearly of intermediate efficiency.

## IV

In comparing the general intensity levels of the flicker contours for different animals we have pointed out (Crozier and Wolf, 1940-41 *a*) that, at  $t_L = 0.50$  (white light), when correction is made for the organism's temperature there is very little difference between the flicker contours for the zebra finch and the turtle *Pseudemys* (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1938-39; Crozier and Wolf, 1939-40). Since the level of  $\tau'$  is more responsive to changes in  $t_L$  with *Pseudemys* than with *Taeniopygia*, the turtle  $t_L = 0.10$  curve would actually fall (for the same body temperature) at an intensity level below that for the zebra finch. In our observations with the house sparrow (*Passer domesticus*) the contour for  $t_L = 0.10$  lies 0.5 log unit below that for the zebra finch, about where the *Pseudemys* curve would be with the temperature correction. Consequently there is nothing very mysterious about the high flicker acuity of birds in general. The relative inefficiency of the red light for the finch is thus all the more striking.

Before discussing this, however, it may be pointed out that if it were mechanically possible to extend the R contour in Fig. 2 by using sufficiently high intensities the R curve would cross the others, its asymptotic maximum being a little higher. In the human data (*cf.* Crozier and Wolf, 1941-42 *b, c*, etc.) the relative separation of the B and R curves is, of course, in general markedly greater at lower ("rod") intensities than at higher—a manifestation of the Purkinje effect, while for test areas entirely within the fovea there is practically no such effect. The (theoretical) crossing of the other curves by the R in the data of Fig. 2 is not exactly the sort of effect which has been in mind in discussions of this point for visually duplex birds (*cf.* Lashley, 1916; Laurens, 1923; van Eck, 1939), but it suggests certain possibilities for the investigation of Purkinje effects in the "cone" curves of other forms, where the crossing could conceivably be more extreme under certain conditions.

The differences between the flicker wavelength functions for man and *Taeniopygia* can be studied most simply by considering the relative magnitudes of the shifts in mean energies for activation of one half the potentially excitable flicker recognition elements, under such conditions that  $F_{max}$  is about the same for each set. The filters used were the same. For the "cone" curves with man (Crozier and Wolf, 1941-42 *c*) B is more effective than V or G, whereas with the bird B is markedly less effective. With man the red is, on the mean energy flux basis, less effective than V or G, or than W. In the bird, however, the R is very much less effective, while in keeping with the "average" quality of white we find W and B about equivalent, and less effective than V, G, or Y, although more potent than R. The data on man used here refer particularly to tests with a  $6^\circ$  square test field centered at the fovea (Crozier and Wolf, 1941-42 *b*). The order of wavelength effectiveness is the same for a quite small field ( $0.6^\circ$  square) within the fovea, as other experiments

have shown, but is modified in other parts of the retina and is to some extent a function of image area as well. If instead of being put upon an energy basis the corresponding data for bird and man are compared with intensities on the scale for photometric brightness, the red (like the white) is still *relatively* much less efficient for the bird, the blue slightly less, and the green slightly more efficient.

In making these comparisons several factors are, of course, not taken into account. The nature of the apparatus used rules out the necessity for an iris correction in the data on man, and the retinal area (absolute, and in relation to full-eye illumination) is not considered although it is different in the bird. The fact that the curves of Fig. 2 are symmetrical, together with the fact that  $\sigma'_{\log I}$  is so nearly the same for all wavelengths, shows that there is no ground for suspecting that in the bird's case there is any complication due to iris contraction. It would be remotely possible, of course, in view of the nature of the probability summation, to have the symmetry of the curve unaffected if the iris opening followed the same law, but in that case it could not be expected that  $\sigma'_{\log I}$  would be independent of  $\lambda$ . The point can be tested with other birds in which the iris opening is more easily observed.

Of greater importance is the fact that in the two sets of measurements no corrections have been made for absorption of light by the ocular media. Although we cannot in any case expect indications from determinations of thresholds to agree entirely with those given by the flicker contours, the close qualitative correspondence between the wavelength intensity functions for responses in man, rat, pigeon, owl (*cf.* Graham and Riggs, 1935; Laurens, 1923; Hamilton and Coleman, 1933; Graham, Kemp, and Riggs, 1935; Hecht and Pirenne, 1940-41) suggests that no gross differences seem to exist in the absorptions of the several ocular media. Since, however, even the wavelength sensitivity function for the *Limulus* visual cell does not differ in any striking way (Graham and Hartline, 1934-35) the force of this conclusion may be questionable. A full analysis of the significance of threshold effects is required before it can really be understood (*cf.* Crozier, 1940*a*). With respect to flicker it is fairly clear that differences between flicker contours are determined by differences in numbers of units activated as well as by differences in the average neural activities of single units under changed conditions. Differences in ocular absorption might be quite complexly involved in modification of these two factors, both as regards threshold effects or amounts of sensory action as well as flicker.

In comparing the flicker contours for man and finch the relative inefficiency of the blue and the marked inefficiency of the red with the finch immediately suggests a differential role of the cone pigment globules. This can be tested. The deduction is that reddish oil globules may here be scanty or even lacking and that these cone globules in the finch may be predominantly greenish yellow. For an account of the several retinal pigments, as found in the chicken,

*cf.* Wald and Zussman, 1938. It has been suggested that in diurnal birds orange and reddish globules are usually found to be abundant (Schultze, 1866; Hess, 1912; Henning, 1920; Erhard, 1924; Blässer, 1936; van Eck, 1939), and that such birds are comparatively blind in the blue and violet. Certainly there is no evidence for anything of the latter sort in the present data.

Our ophthalmoscopic observations of the zebra finch eye agree rather well with Wood's account for members of the related Fringillidae (Wood, 1917, plate LIV); the eyeground is bright blue-green gray, with bright dots, and with no differentiated red region. When the freshly teased retina was viewed under the microscope it was found (after the conclusion of the flicker observations) that approximately 15 per cent of the oil globules were bright red, the remainder greenish; a few of the latter were bluish green, some yellowish green, and a much smaller number definitely yellow. The small proportion of reddish globules in this diurnal bird represents a rather sharp difference from what has been customarily described, but is quite consistent with the deduction made from the properties of the flicker contours. It should be remarked also that the differentiation of the definitely red globules is quite sharp in the case of the zebra finch, there being no orange globules.

#### SUMMARY

With lights of different spectral compositions filtered from a white, the flicker response contours for the zebra finch are found to exhibit the same general kind of relationship between flicker excitation and wavelength as is found in the case of man ("cone" contours), with the same filters. The flicker contours for the zebra finch are simplex; the retina contains no rods. On a relative energy scale, with a flash cycle of fixed light-time percentage (10 per cent) the curve for yellow almost coincides with that for the white, the curve for red lies at much higher intensities, and the curves for blue-green and violet fall below that for the white by amounts increasing in that order. The maxima to which the curves rise and the slope constants are very nearly the same for all the spectral regions. For the bird the blue was a little less efficient, the green a little more efficient, and the red very much less efficient than in the case of man. It was deduced that in the retina of this diurnal bird the number of red oil globules should be comparatively small and that most of the globules should be greenish yellow. This was confirmed by direct examination.

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# COUNTERACTING THE RETARDING AND INHIBITORY EFFECTS OF STRONG ULTRAVIOLET ON FUCUS EGGS BY WHITE LIGHT\*

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## INTRODUCTION

It has been shown that moderate dosages of unilateral ultraviolet light cause rhizoids to form on the non-irradiated sides of *Fucus* eggs (Whitaker, 1941; Whitaker, 1942). Thus 50 ergs per mm.<sup>2</sup> of  $\lambda$  2537 Å cause more than 98 per cent of the eggs in a population to respond (Whitaker, 1942). Dosages of this order of magnitude have no appreciable effect on the developmental rate, but dosages of 20,000 to 50,000 ergs per mm.<sup>2</sup> very considerably retard and inhibit development (Whitaker, 1942).

The addition of  $\beta$ -indole acetic acid to the medium after eggs have been irradiated with 20,000–50,000 ergs per mm.<sup>2</sup>  $\lambda$  2537 Å does not revive the eggs or cause them to recover from the retarding and inhibiting effects of the ultraviolet light. If the sea water medium in which eggs are strongly irradiated and then reared is acidified from pH 8.0 to pH 6.0, the sensitivity of the eggs to the ultraviolet is decreased (Whitaker, 1942). The present experiments were undertaken to test the effect of white light upon the sensitivity of the eggs to strong dosages of ultraviolet.

## Material and Method

*Fucus furcatus* was collected at Moss Beach and at Pescadero Point, California, during the months of April, May, and June, 1941. Gametes were obtained and fertilized in a manner previously described (Whitaker, 1942; Whitaker, 1936). Filtered sea water (specific gravity 1.025–1.027) at pH 7.9–8.1 was used as the medium throughout. The eggs were fertilized and reared in a constant temperature room at  $15 \pm 1/4^\circ\text{C}$ ., and were shielded from all light except the experimental exposures, and brief exposures to dim red light.

The eggs were irradiated with ultraviolet light in 4 clear fused quartz rectangular culture vessels,  $50 \times 25 \times 15$  mm. These vessels were made of polished stock 1 mm. thick and they have good optical properties. The eggs were arranged on plate glass slabs within the quartz vessels so that they were in two rows. One row

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was above and behind the other so that there was no eclipsing, and eggs were at least 5 egg diameters apart in the row. The radiation passed through  $9 \pm 1$  mm. of sea water before reaching the eggs.

A Westinghouse sterilamp was used as the source of ultraviolet. It is a gas tube mercury resonance lamp, and more than 90 per cent of the radiant energy is of the wave-length 2537 Å. An appreciable amount of  $\lambda 1800$  Å is produced in the gas space, but almost all of this ozone producing frequency is absorbed in the corex glass tube of the lamp. Small amounts of  $\lambda 3130$  and  $3660$  Å are emitted, as well as dim visible light of bluish color. Practically no heat is produced. The intensity of ultraviolet wave-lengths shorter than  $3200$  Å was measured by means of a Hanovia ultraviolet meter. The intensity of the lamp did not vary more than 6 per cent at most during a run. Intensity measurements were made periodically during each run, and the duration of the exposure was adjusted to compensate for variations in intensity, to give the desired total dosage. The eggs were placed 6 inches from the tube of the sterilamp and the rate of application of the ultraviolet energy to the eggs was approximately 525–550 ergs per mm.<sup>2</sup> per minute. The absorption of the ultraviolet in passing through 9 mm. of sea water to reach the eggs is minor and has been neglected in calculating dosage. The mid-point of the period of exposure to ultraviolet occurred in all cases very nearly at 8 hours after fertilization. The duration of the exposure to ultraviolet was very nearly 36 minutes for 20,000 ergs, 66 minutes for 35,000 ergs, and 95 minutes for 50,000 ergs.

Due to the delay in development caused by the ultraviolet it was necessary to extend observations over a number of days. Therefore, in order to free the quartz vessels for starting new experiments, after irradiation with ultraviolet the eggs were transferred on the glass slabs from the quartz vessels into rectangular Petri dishes for rearing with or without white light. Although this transfer was carried out with great care, the eggs may have moved somewhat, and therefore little consideration has been given in these experiments to the direction of rhizoid formation. Incomplete evidence suggests that eggs do not necessarily form rhizoids on the sides away from strong doses of  $\lambda 2537$  Å.

## RESULTS

In the first series of experiments, the eggs in half the vessels were exposed to white light from an ordinary frosted 75 watt bulb at 1 meter distance. This exposure to white light began immediately after termination of the irradiation with ultraviolet and transfer of the eggs to the Petri dishes, and continued until the end of the experiment. The same sides of the eggs were exposed to ultraviolet and to white light, except for any movement of the eggs during transfer (see Method). The white light passed through a water cell, and a fan caused air to flow from the eggs toward the light to minimize temperature effects.

The object of this first series of experiments was to see whether the white light would to some extent revive the eggs or cause them to recover from the retarding and inhibitory effects of the ultraviolet, when applied only after termination of the ultraviolet treatment. A part of the results is shown in

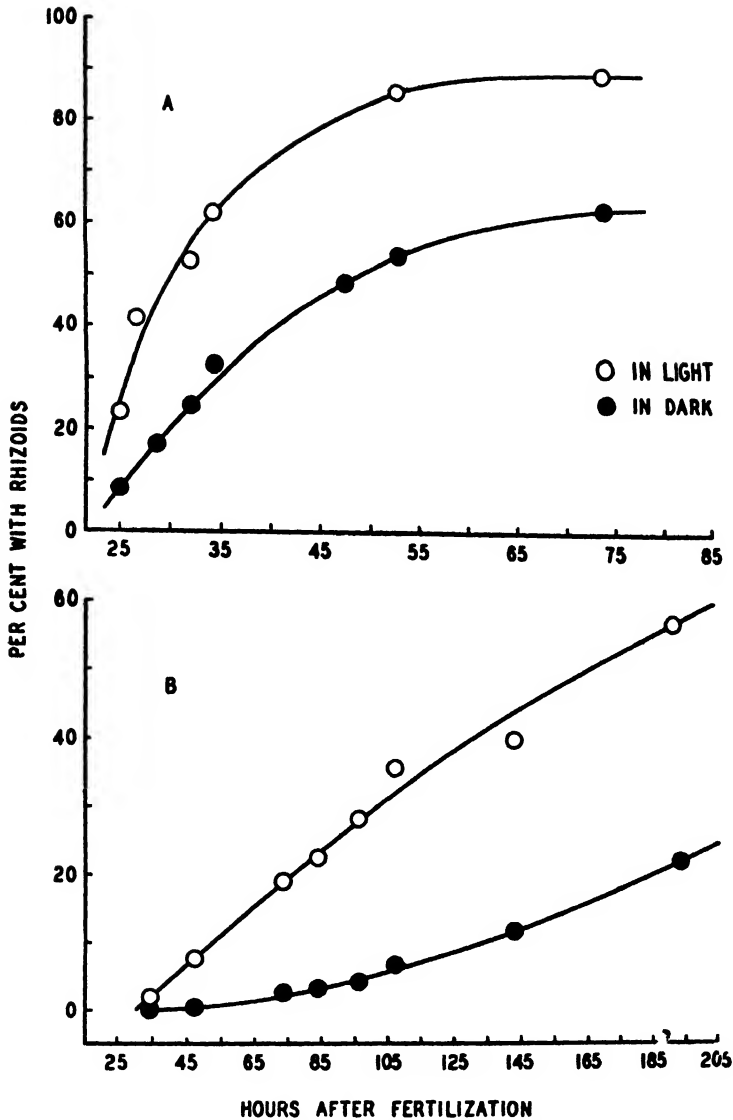


FIG. 1. Typical results showing effect of white light in accelerating rhizoid formation after eggs have been exposed to retarding and inhibiting dosages of ultraviolet. The white light was turned on after termination of the ultraviolet treatment at about 8.4 hours after fertilization. In A, the eggs received 20,000 ergs per mm.<sup>2</sup> ultraviolet; 446 eggs were reared in the light and 393 in darkness. In B, the ultraviolet dosage was 35,000 ergs per mm.<sup>2</sup>; 398 eggs were reared in the light and 344 in darkness.



Fig. 1. Fig. 1 A shows the results of a typical experiment in which 20,000 ergs per mm.<sup>2</sup> ultraviolet were applied. Two other similar experiments involving 201 and 300 eggs reared in the white light, and 296 and 270 eggs reared in the dark, respectively, gave results very much like those shown in Fig. 1 A, but the differences between the rates of development in the white light and in the dark were somewhat greater. Fig. 1 B shows the results of an experiment in which 35,000 ergs per mm.<sup>2</sup> ultraviolet were applied. The retardation of development is greater in this case, but the effect of the white light is very marked in reducing the retardation. Another similar experiment, involving 320 eggs reared in the dark and 280 reared in the white light, gave similar results, although the effect of the white light was not quite so great. After 50,000 ergs per mm.<sup>2</sup> ultraviolet, the developmental delay was very great. Most of the eggs did not develop at all before cytolizing some days after the irradiation with ultraviolet. However, in two experiments with 50,000 ergs per mm.<sup>2</sup> ultraviolet, more eggs formed rhizoids in the white light than in the dark, and they formed them sooner.

It is clear from these results that the rhizoids form much sooner in the white light after the eggs have been exposed to ultraviolet. However, it has already been shown that normal *Fucus* eggs form rhizoids somewhat sooner in the light than in the dark (Whitaker, 1936). In the present experiments it is therefore of interest to find out how much acceleration of rhizoid formation can be attributed to the effects of the white light quite aside from the effects of ultraviolet. For this purpose, in a second series of experiments, eggs were illuminated with white light from the 75 watt bulb at 1 meter distance beginning at 8.4 hours after fertilization and continuing to the end of the experiment. The white light was also turned on at about 8.4 hours after fertilization in the first series of experiments. No ultraviolet was used in this second series of experiments. Two experiments were carried out, involving 4 vessels each, and the eggs in half the vessels served as dark controls. The rhizoids formed sooner in the light, but the speed up is not more than 2.5 hours at most, under these conditions. The speed up in the light is probably somewhat exaggerated as measured, since the light came from one side and caused the rhizoids to form on the opposite sides of the eggs where they could be seen from above at the very earliest stages of formation. In the two experiments, the time at which 50 per cent of the population formed rhizoids was earlier in the light by  $1\frac{1}{4}$  hours in one case, and by  $1\frac{1}{2}$  hours in the other. Half of the eggs had formed rhizoids in the dark at 16 hours after fertilization. This acceleration is small compared with that caused by the white light after strong dosages of ultraviolet.

A third series of experiments was undertaken to see if a strong white light applied during the time of exposure to ultraviolet would exert a protective effect upon the eggs. An ordinary 200 watt bulb was placed at a distance of 14 inches from the eggs in half the vessels. The white light passed through a water cell

to absorb heat, and the same sides of the eggs received both the ultraviolet and the white light. The white light was turned on in all cases 4 minutes before the ultraviolet, but the ultraviolet and the white light were turned off at the same time. Thereafter the eggs developed in darkness except for brief exposures to dim red light to make counts. In two experiments the eggs were exposed to 20,000 ergs per mm.<sup>2</sup> ultraviolet. In both cases the exposure lasted 36 minutes, its mid-point occurring at 8 hours after fertilization. The results of one of these are shown in Fig. 2. The results of the other, involving 200 eggs ex-

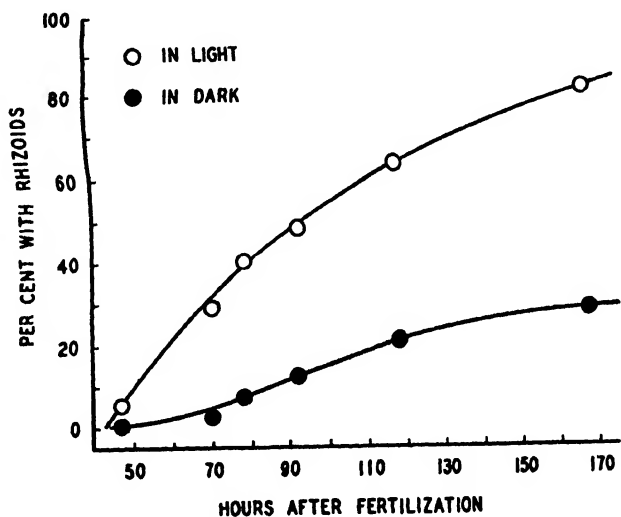


FIG. 2. Results of an experiment showing the protective effect of exposure to strong white light during exposure to retarding ultraviolet (20,000 ergs per mm.<sup>2</sup>). Two vessels containing a total of 375 eggs were exposed to white light and ultraviolet; the averaged results are shown with open circles. Two control vessels containing 398 eggs were exposed to ultraviolet only; the results are shown with solid circles.

posed to white light and 300 controls, are essentially similar although the rate of rhizoid formation in the light was not quite so great. A third experiment was carried out with 35,000 ergs per mm.<sup>2</sup> ultraviolet applied during 66 minutes (33 minutes on either side of 8 hours after fertilization). The development was very greatly inhibited by the ultraviolet in this experiment, but at 170 hours after fertilization rhizoids had formed on 7 per cent of 280 eggs that received white light with the ultraviolet, and on 1½ per cent of 270 eggs that received ultraviolet only. The concurrent white light thus appears to reduce the inhibition as well as the retardation of rhizoid formation.

## DISCUSSION

From the results of the first series of experiments (Fig. 1), it is clear that white light applied after strong dosages of ultraviolet counteracts the retarding action of the ultraviolet on development to a very considerable extent. Since white light tends to speed up the rate of rhizoid development in normal *Fucus* eggs that have not been subjected to ultraviolet light (Whitaker, 1936), it is of interest to compare the accelerating effect of white light with and without ultraviolet treatment. The results of the second series of experiments show that the white light does not speed up the rhizoid formation, in the absence of ultraviolet effects, more than about 2.5 hours at most, under the conditions of the experiments. After eggs have been retarded by heavy dosages of ultraviolet, the white light has a much greater accelerating effect than this, as may be seen in Fig. 1. In Fig. 1 B, for example, 20 per cent of the population reared in white light formed rhizoids more than 100 hours before the same percentage of the population reared in the dark. After the strongest dosages of ultraviolet (e.g., 50,000 ergs per mm.<sup>2</sup>), which completely inhibit rhizoid formation in most of the eggs, the white light appears also to increase the percentage of eggs that form rhizoids before cytolizing, although this effect is not as well established as the increase in rate of rhizoid formation.

The third series of experiments shows that strong white light shining on the eggs at the same time they are receiving heavy dosages of ultraviolet greatly protects the eggs from the retarding and inhibiting effects of the ultraviolet (Fig. 2). This protective effect appears to be even more marked than the recovery effect shown in the first series of experiments, although the white light was on so briefly that it cannot have had much of the type of effect shown in the second series of experiments. The white light was turned on 4 minutes earlier than the ultraviolet, in the third series of experiments, so that photosynthesis might be well established when the ultraviolet began to fall on the cells.

Until monochromatic bands or limited regions of the spectrum are tested, there is of course no basis for a definite opinion about what frequencies are effective or about the means of action of the white light. If the longer wavelengths of the visible spectrum are effective, it might be supposed that photosynthesis is involved.

## SUMMARY

1. Strong dosages (20,000–50,000 ergs per mm.<sup>2</sup>) of ultraviolet light, predominantly of the wave-length 2537 Å, greatly retard and inhibit the development of rhizoids in *Fucus* eggs irradiated at about 8 hours after fertilization.

2. If white light shines on the eggs after the irradiation by ultraviolet is terminated, the white light causes a considerable degree of recovery from the retarding and inhibiting effects.

3. If strong white light shines on the eggs during the ultraviolet irradiation, its effect is even more marked in protecting the cells from the damaging effects of the ultraviolet.

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# THE ACTIVITY OF YEAST INVERTASE AS A FUNCTION OF OXIDATION-REDUCTION POTENTIAL

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The activity of many enzymes is very appreciably modified by oxidizing or reducing agents. Such enzymes as urease (Hellerman, 1939; Sizer and Tytell, 1941), cathepsin and papain and papain-like enzymes (Hellerman, 1937; Greenberg and Winnick, 1940), carbonic anhydrase (Kiese and Hastings, 1940), succinic dehydrogenase (Hopkins, *et al.*, 1938), triosephosphate dehydrogenase (Rapkine, 1938), and glycerol oxidase (Barron, 1940) are activated by reducing agents and inactivated by oxidizing agents. These effects have been interpreted in terms of the configuration of thiol groups in the enzyme molecule; when the sulfur is in the reduced -SH form the enzyme is active, when in the oxidized -SS- form the enzyme is inactive. Fruton and Bergmann (1940) and Irving *et al.* (1941) believe that the activation of papain and cathepsin cannot be explained by this hypothesis, and state that activation is a two-step process involving the formation of an addition complex between the activator and the enzyme. Sizer and Tytell (1941) reported, in the case of crystalline urease, that activity is a continuous function of oxidation-reduction potential with an optimum activity at  $E_h = +150$  mv. and a gradual decrease in activity at lower as well as higher potentials. It seems very important for the interpretation of enzyme action to find out whether variation of enzyme activity with oxidation-reduction potential is characteristic of other enzyme systems as well. Many enzyme systems do not appear to be sensitive to oxidants or reductants, possibly due to the presence of stabilizers accompanying the enzyme (*cf.* Irving *et al.*, 1941) or because of the greater stability of the enzyme molecule. Such an enzyme is yeast invertase which, although relatively unaffected by weak oxidants or reductants (Ito and Obo, 1939), is inactivated by a wide variety of other agents such as certain metal salts,  $I_2$ ,  $HNO_3$ , phenylhydrazine (Myrbäck, 1926), by nitrophenols, quinine, and its derivatives (Rona and Bach, 1921), aniline (Euler and Svanberg, 1921), alkaloids (Rona *et al.*, 1924; Mezzadrolì and Amati, 1933), alcohols (Colin and Chaudin, 1931), certain dyes and narcotics (Baur, 1939), ascorbic acid (Klodnitskaya and Strachiskii, 1938). Using

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this enzyme as an example of those not affected by mild oxidants or reductants, a systematic study of the relationship of invertase activity to oxidation-reduction potential has been made.

### Methods

The kinetics of the yeast invertase-sucrose system were studied by measuring the amount of invert sugar liberated during the hydrolysis, by utilizing for this purpose Sumner's dinitrosalicylic acid reagent for reducing sugars (Sumner, 1925; Sumner and Howell, 1935), which has been found satisfactory for invertase studies (Sizer, 1938 *a*, 1938 *b*). Using this technique it was found that the reaction follows a smooth curvilinear course, and that the reaction is unimolecular for the first third of the inversion (Sizer, 1938 *a*, 1938 *b*). In view of the uniform kinetics during the early portion of the inversion, the amount of invert sugar liberated in a given time can be taken as a measure of rate. In preliminary experiments the 2 minute determination of invert sugar was subtracted from the 10 minute one. If the 2 minute determination was neglected, however, the results were relatively the same, so in all subsequent experiments duplicate samples of the digest were taken for the analysis of invert sugar after the inversion had proceeded for 10 minutes.

A number of samples of commercial invertase (Digestive Ferments Co., Wallerstein Laboratories) was used as well as fresh and powdered bakers' yeast (Fleischmann) and brewers' top yeast (Haffenreffer), and several invertase preparations of varying degrees of purity prepared from bakers' yeast according to Lutz and Nelson (1934). In all cases the invertase was dissolved in distilled water and diluted to such a concentration that it would liberate 10–20 mg. invert sugar per ml. at  $30 \pm 0.01^\circ\text{C}$ . in 10 minutes. 1 ml. of the enzyme solution was added to 1 ml. oxidant or reductant (usually 0.1 *M*) and incubated for 5 minutes. To this solution were then added 8 ml. 6 per cent sucrose dissolved in phthalate buffer, pH 4.6 (optimum pH for invertase). After the 10 minute inversion period duplicate samples were taken, and the amount of reducing sugars was determined colorimetrically after treatment with the dinitrosalicylic acid reagent. In all preliminary experiments the Duboscq colorimeter was used, but since more quantitative data could be obtained with the Spekker photoelectric absorptiometer, all subsequent work was done using this instrument. The number 6 light blue filter (maximum transmission at  $\lambda = 4810 \text{ \AA}^1$ ) was used. The standard solution contained 20 mg. glucose and was treated in the same manner as the experimental solutions.

The addition of the enzyme plus oxidant or reductant to the sucrose solution did not appreciably modify the pH of the solution which was  $4.63 \pm 0.03$  in all cases except for the solution containing thioglycolic acid where the pH was 4.24. A control which contained distilled water instead of the oxidant or reductant was run with every five experimental tubes, and the activity of the invertase in the presence of oxidant or reductant was expressed as a percentage of the control.

The oxidation-reduction potential of the digest was measured at room temperature after the 10 minute inversion using the Beckman pH- $E_h$  meter. The  $E_h$  of the solutions containing  $\text{Na}_2\text{S}_2\text{O}_4$  or  $\text{KMnO}_4$  was unstable and was measured immediately

<sup>1</sup> Measured with the Hardy recording photoelectric spectrophotometer.

on duplicate digests instead of after the 10 minute interval. Solutions of the unstable oxidants or reductants were freshly prepared just before use.

### *Experimental and Results*

Reaction rate was measured by the amount of invert sugar formed in 10 minutes, and was expressed as a percentage of the rate of the control, which contained distilled water instead of oxidant or reductant. A search was made for oxidants or reductants suitable for poisoning the system at the desired oxidation-reduction potential. In most cases these compounds could be used in a final concentration of 0.01 M without exerting any effect which might be ascribed to the concentration of salt employed. More dilute solutions than this of  $\text{KMnO}_4$  and I-KI had to be used in order to avoid inhibiting effects related to concentration. Preliminary experiments on commercial invertase samples using the Duboscq colorimeter indicated that there was little effect upon invertase activity of changing the  $E_h$  of the digest from  $-270$  to  $+600$  mv., but above  $+600$  mv. the activity decreased rapidly.

Quantitative studies, using the photoelectric absorptiometer for the colorimetric determination of reducing sugar, were made on Wallerstein's red label invertase scales, using fifteen different oxidizing or reducing solutions to poise the oxidation-reduction potential of the digest in the range from  $-270$  to  $+1,000$  mv. The activity of invertase in the presence of each of these solutions was measured on 10 different days and an average of the data on each solution was taken. The average activity was then plotted against the average oxidation-reduction potential as shown in Fig. 1. Despite the appreciable scatter of the points, it is apparent that invertase activity is independent of  $E_h$  up to  $+400$  mv., above which the activity falls sharply reaching 0 at  $E_h = 1,000$  mv. The points for invertase plus  $\text{H}_2$ -Pt asbestos or 0.01 M  $\text{Na}_2\text{S}_2\text{O}_4$  are probably not significantly below the curve, since in many of the individual experiments the corresponding activities were 100 per cent. The point for invertase plus 0.00001 M I-KI, is very low; this may be related to a toxic as well as an oxidizing effect of iodine.

A number of different samples of commercial invertase were then studied to determine the relationship of the  $E_h$ -activity curve to the purity, source, and method of preparation of the enzyme. Difco liquid invertase, Wallerstein's blue label, green label, and liquid invertase preparations were used. The data with all four commercial samples are very similar, so only one characteristic curve is presented in Fig. 2 (curve A). The curve is very similar to that of Fig. 1, indicating a constant activity over the  $E_h$  range from  $-270$  to  $+400$  mv. and a rapid decrease in activity above  $+400$  mv.

### *Experiments with Highly Purified Invertase*

From the preceding experiments with different commercial preparations of invertase it appears that the  $E_h$ -activity curve is independent of the source and



type of preparation used. Since these samples of invertase may contain natural activators or stabilizers, the  $E_h$ -activity curve could be explained in

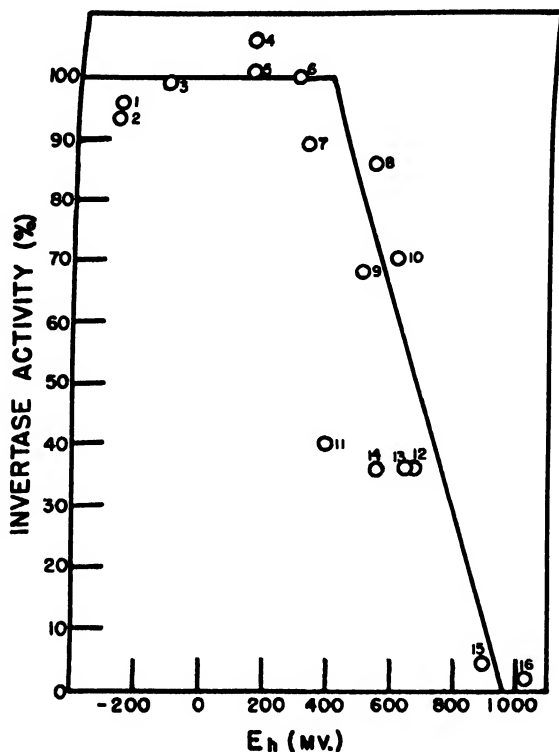


FIG. 1. Per cent invertase activity is plotted as a function of the oxidation-reduction potential of the digest. The digest contained 0.005 per cent Wallerstein's red label invertase, 5 per cent sucrose, phthalate buffer pH 4.6, and one of the following:

- |                              |                        |
|------------------------------|------------------------|
| 1. $H_2$ + Pt asbestos       | 9. 0.01 M $K_2Cr_2O_7$ |
| 2. 0.01 M $Na_2S_2O_4$       | 10. 0.01 M $H_2O_2$    |
| 3. 0.001 M $Na_2S_2O_4$      | 11. 0.00001 M I-KI     |
| 4. 0.01 M cysteine           | 12. 0.0001 M I-KI      |
| 5. 0.01 M thioglycollic acid | 13. 0.001 M I-KI       |
| 6. Water                     | 14. 0.00001 M $KMnO_4$ |
| 7. 0.01 M $K_4Fe(CN)_6$      | 15. 0.0001 M $KMnO_4$  |
| 8. 0.01 M $K_3Fe(CN)_6$      | 16. 0.001 M $KMnO_4$   |

terms of the effects of the oxidants or reductants on such impurities rather than on the invertase itself. With impure papain (Irving, *et al.*, 1941) and impure urease (Sizer and Tytell, 1941) such contaminants have been shown to be important in activation-inactivation phenomena. In view of this evidence it

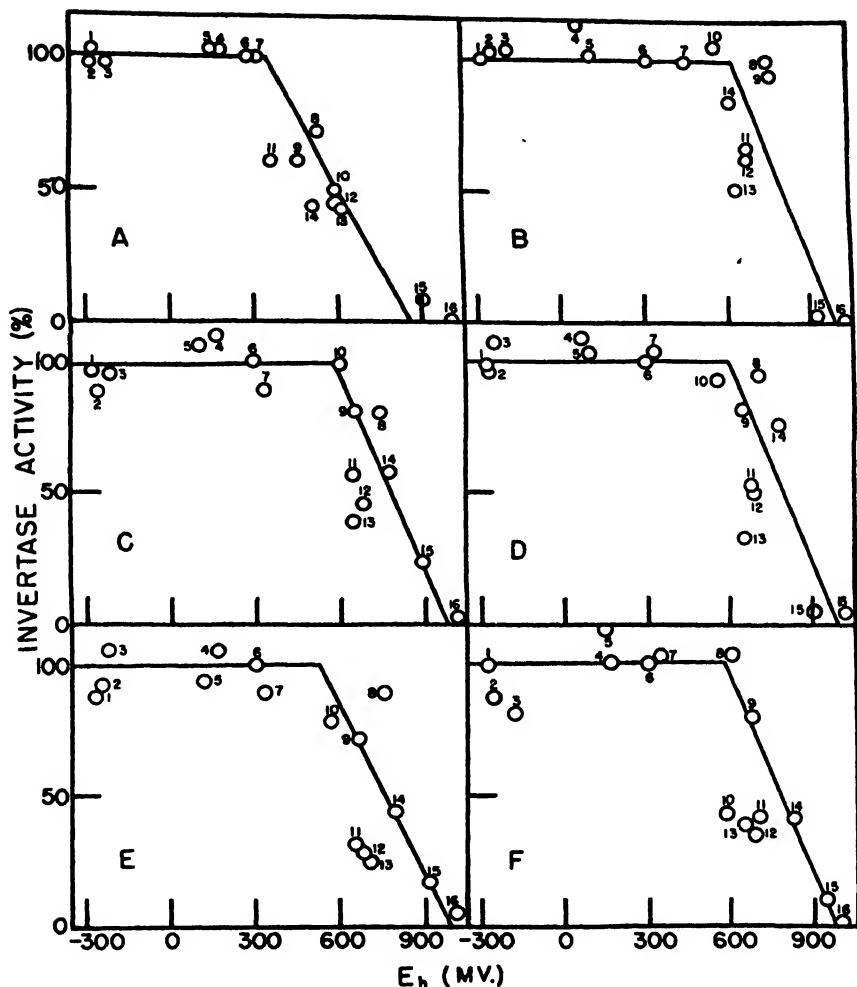


FIG. 2. Per cent invertase activity is plotted against the oxidation-reduction potential of the digest. The numbers refer to the same oxidants or reductants as in Fig. 1. Different invertase preparations were used for each curve. A, Wallerstein green label invertase. B, fresh Fleischmann's bakers' yeast. C, dried, powdered Fleischmann's bakers' yeast. D, filtrate of toluene autolysate of yeast. E, kaolin eluate of invertase prepared according to Lutz and Nelson. F, dialyzed  $(\text{NH}_4)_2\text{SO}_4$  filtrate of highly purified invertase prepared according to Lutz and Nelson.

seemed advisable to determine the possible rôle of contaminants by studying the  $E_h$ -activity curve of invertase as a function of enzyme purity.

For impure invertase use was made of untreated fresh and powdered bakers' yeast. In the former practically 100 per cent of the yeast cells were alive (as

determined by the differential methylene blue staining technique, Nelson, *et al.*, 1932), while in the dried yeast about 2/3 of the cells were dead. The corresponding  $E_h$ -activity curves for these two sources of invertase are shown in Fig. 2, curves B and C. The curves are similar to those for commercial invertase, although the break seems to occur at a somewhat higher  $E_h$ . The question arises in the case of the fresh yeast as to whether or not the action of the reagent on the enzyme inside the cell is preceded by the death of the cell. Experiments on this problem indicated that only in the presence of  $\text{KMnO}_4$  and I-KI, is the mortality of the cells appreciable in a period of 15 minutes. Experiments were also performed on brewers' yeast and it was found that the  $E_h$ -activity curve was very similar to that for bakers' yeast.

Invertase was prepared from bakers' yeast<sup>2</sup> by the method of Lutz and Nelson (1934). By this method a 500-fold increase in activity above that of the original toluene autolysate was obtained. At each step in the purification samples of the invertase were taken in order to determine how the  $E_h$ -activity curve changed as the purity of the enzyme increased. Only three typical curves for yeast invertase in three different stages of purification are shown in Fig. 2; curve D is for the filtrate obtained after autolysis of the yeast with toluene, curve E for the eluate after kaolin absorption, curve F for the filtrate after saturation with  $(\text{NH}_4)_2\text{SO}_4$  and dialysis. All the curves are very similar with no change in activity between  $-270$  and  $+600$  mv.; above the latter the activity falls rapidly becoming 0 at  $E_h = +1,000$  mv. The most highly purified preparations seemed somewhat less stable, and were slightly inactivated by the  $0.01$  M  $\text{Na}_2\text{S}_2\text{O}_4$ . The fact that the  $E_h$ -activity curve is essentially independent of invertase purity makes it seem unlikely that the curve can be explained in terms of effects on natural activators, stabilizers, or inhibitors which accompany the invertase. This conclusion must be accepted with caution since pure crystalline invertase was not used, and since it is not impossible that during the purification of invertase these substances possibly concerned with the  $E_h$ -activity curve maintained their same relative concentrations.

#### *Other Oxidizing or Reducing Compounds*

Oxidizing or reducing agents cannot be used indiscriminately to poise the invertase system, since some of them have a specific toxic action in addition to  $E_h$  effects. In this category are such substances as  $\text{Br}_2$ ,  $\text{Na}_2\text{S}$ ,  $\text{FeCl}_3$ , ascorbic acid, and iodoacetic acid. On the other hand many other compounds prove quite satisfactory and the typical  $E_h$ -activity curve can be obtained when they are used to poise the digest (Fig. 3, curve A).

<sup>2</sup> The author wishes to acknowledge the assistance of Mr. Herbert Jaffe in the purification of the invertase.

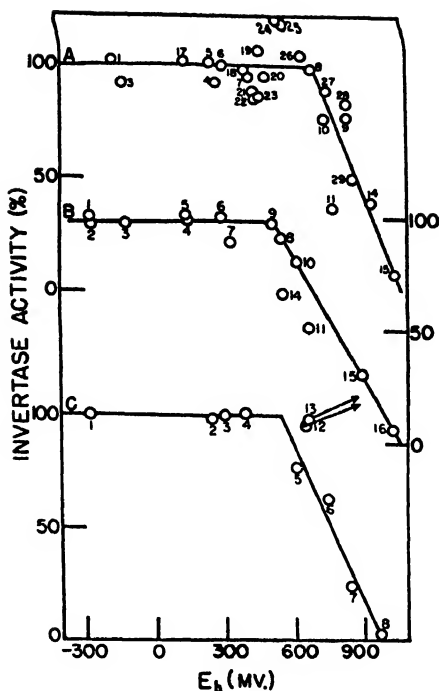


FIG. 3. Per cent invertase activity is plotted against the oxidation-reduction potential of the digest.

Curve A. Invertase purified according to Lutz and Nelson. The oxidants or reductants are the same as in Fig. 1 plus the following:

- |                                        |                                           |
|----------------------------------------|-------------------------------------------|
| 17. 0.1 saturated $\text{H}_2\text{S}$ | 23. 0.01 M KCNS                           |
| 18. 0.01 M methionine                  | 24. 0.01 M KCN                            |
| 19. 0.01 M glutathione                 | 25. Saturated quinone                     |
| 20. 0.01 M $\text{CeCl}_3$             | 26. Saturated cystine                     |
| 21. 0.01 M $\text{FeCl}_2$             | 27. 0.01 M $\text{Na}_2\text{SeO}_4$      |
| 22. 0.01 M $\text{Na}_2\text{SeO}_3$   | 28. 0.01 M $\text{CrCl}_3$                |
|                                        | 29. 0.00001 M $\text{Ba}(\text{MrO}_4)_2$ |

Curve B. 0.008 per cent Wallerstein red label invertase. The oxidant or reductant was added to the enzyme, then removed by dialysis before the sucrose solution was added. Symbols are the same as in Fig. 1; the ordinate scale is on the right.

Curve C. 0.008 per cent Wallerstein red label invertase, 5 per cent sucrose, plus one of the following:

- |                                              |                                                                         |
|----------------------------------------------|-------------------------------------------------------------------------|
| 1. $\text{H}_2$ -Pt asbestos                 | 5. 0.0001 M $\text{KMnO}_4$ + 0.0009 $\text{K}_4\text{Fe}(\text{CN})_6$ |
| 2. 0.01 M thioglycolic acid                  | 6. 0.0001 M $\text{KMnO}_4$ + 0.0007 $\text{K}_4\text{Fe}(\text{CN})_6$ |
| 3. Water                                     | 7. 0.0001 M $\text{KMnO}_4$ + 0.0004 $\text{K}_4\text{Fe}(\text{CN})_6$ |
| 4. 0.01 M $\text{K}_4\text{Fe}(\text{CN})_6$ | 8. 0.0001 M $\text{KMnO}_4$                                             |

*Do  $E_h$  Changes Act upon the Enzyme or Substrate?*

The oxidant or reductant was incubated with the invertase for 5 minutes, the solution then placed in a cellophane sausage casing (Central Scientific Co.), and the oxidant or reductant removed by dialyzing for 1 hour under turbulent tap water (chemical tests showed this time to be sufficient for the removal of these substances). The invertase activity of the solution was then measured in the usual manner (Fig. 3, curve B). This curve is very similar to the others, and indicates that the oxidant or reductant acts solely on the enzyme, not on its substrate.

TABLE I

Activity of invertase after treatment with oxidizing agents, followed by dialysis. Platinized asbestos was then added to all the enzyme solutions and the invertase activity was determined with and without the addition of hydrogen.

Oxidant	Hydrogen added	Invertase activity
		<i>per cent</i>
Water, control	No	100
0.0001 M $\text{KMnO}_4$	No	6
" " "	Yes	6
0.0001 M I-KI	No	0
" " "	Yes	0
0.01 M $\text{K}_3\text{Fe}(\text{CN})_6$	No	84
" " "	Yes	62
0.01 M $\text{K}_2\text{Cr}_2\text{O}_7$	No	84
" " "	Yes	91
0.01 M $\text{H}_2\text{O}_2$	No	84
" " "	Yes	85

*Reversibility of the Inhibiting Action of Oxidants*

It is apparent from Fig. 3, curve B, that the effects of oxidizing agents are not readily reversible, since the invertase did not regain its activity after these inhibitors were removed by dialysis. The problem of reactivation was also studied by bubbling  $\text{H}_2$  (activated by platinized asbestos) into the invertase solution freed of oxidants by dialysis. By reducing the  $E_h$  to a low value it seemed possible that reactivation might be effected. Examination of Table I, however, reveals that no reactivation was obtained by this method. Reactivation was also attempted by adding the reducing agents,  $\text{Na}_2\text{S}_2\text{O}_4$ ,  $\text{Na}_4\text{S}_2\text{O}_3$ , and  $\text{K}_4\text{Fe}(\text{CN})_6$ , to the invertase which had been incubated for 5 minutes with the oxidant. Although the oxidant was not previously removed, the addition of the reducing agent brought about a marked fall in  $E_h$ . In the series of studies made on the reversal by reductants of invertase inactivation by each of the oxidants there was little or no reactivation. It appears from this in-

vestigation that the inactivation of invertase by oxidants is essentially irreversible.

### *Toxicity of Oxidants*

The inactivation of invertase by oxidants may be due to their high oxidation-reduction potential or may be caused by a specific toxicity of the oxidant unrelated to  $E_h$ . The fit of points obtained with different oxidants to a single smooth curve (Figs. 1-3) may be taken as evidence of the former hypothesis. Additional evidence on this problem can be obtained by adding a reductant ( $K_4Fe(CN)_6$ ,  $Na_2S_2O_3$ , and  $Na_2S_2O_4$  were used) to the various oxidants before adding the enzyme, thereby bringing about a corresponding fall in  $E_h$ . Since the concentration of the oxidant is held constant in all these experiments, it may be argued that any observed increase in activity (as compared with the activity in the presence of oxidant alone) must be due to the fall in potential. From this it follows that the original effect of the oxidant could be attributed to the high  $E_h$ , and not to a specific toxic action unrelated to  $E_h$ . A typical experiment to illustrate this point is shown in Fig. 3, curve C, where adding increasing amounts of ferrocyanide to a permanganate solution effects a corresponding decrease in  $E_h$ , and an increase in the activity of the invertase subsequently added to the mixture. These results cannot be explained on the basis that the addition of ferrocyanide has caused the disappearance of the "toxic"  $MnO_4^-$  ions, because chemical analysis indicates that the majority of the  $MnO_4^-$  ions are still present in the solution.

Essentially similar results to those with  $KMnO_4$  were obtained for  $I-KI$ ,  $K_2Cr_2O_7$ ,  $H_2O_2$ , and  $K_3Fe(CN)_6$ , for when  $K_4Fe(CN)_6$ ,  $Na_2S_2O_3$ , or  $Na_2S_2O_4$  were added to the oxidant before the invertase was added, the expected increased activity was obtained in most cases. These results suggest that the inhibiting action of oxidants on invertase is primarily related to their high oxidation-reduction potentials rather than to a specific toxicity unrelated to their oxidizing powers.

### DISCUSSION

It is apparent from this investigation that invertase is a stable enzyme over a wide range of oxidation-reduction potential, but loses its activity in strong oxidizing solutions. It might be thought that the inactivating action of such oxidizing solutions is analogous to the more violent conditions characteristic of the oxidative degradation of proteins used in total nitrogen determinations. Such is apparently not the case, however, since the dilute 0.01 M solutions of oxidants acting for only 15 minutes at 25°C. brought about no apparent protein degradation. While this oxidative degradation of invertase by oxidants seems to be ruled out, other explanations of their action can be suggested. An explanation in terms of changes in substituent sulfur groupings in the

enzyme is unlikely since free thiol groups can usually be oxidized at lower potentials than those required to inactivate invertase (Hellerman, 1939; Sizer and Tytell, 1941). Strong oxidants might react with constituent amino acids of invertase such as tyrosine, tryptophane, or histidine and in this way inactivate the enzyme. This would be analogous to pepsin inactivation by the iodination of tryrosine in the pepsin molecule (Herriott, 1937). Another plausible explanation for invertase inactivation by oxidants is that these agents act by denaturing the protein enzyme. Northrop (1939) has shown that denaturation of certain proteases is always accompanied by loss in enzyme activity. Reducing agents apparently do not denature proteins (Mirsky, 1938), but the denaturation of proteins by oxidants has not yet been sufficiently studied to state whether or not this mechanism is likely.

#### SUMMARY

The activity of yeast invertase as a function of oxidation-reduction potential has been investigated using a large number of oxidants and reductants. The activity is constant over the range of  $E_h$  from  $-270$  to  $+600$  mv., but above  $E_h = +600$  mv. there is a sharp decrease in activity reaching 0 at  $E_h = +1,000$  mv. The inhibiting action of strong oxidants is upon the enzyme rather than on the substrate and appears to be essentially irreversible. Experiments indicate that the inhibiting action of strong oxidants on invertase is primarily related to their high oxidation-reduction potential rather than to a specific toxic action unrelated to  $E_h$ . The effects of oxidation-reduction potential upon invertase activity are independent of the purity of the enzyme, since they are the same for commercial invertases, fresh bakers' yeast, powdered bakers' yeast, brewers' yeast, and highly purified invertase. Possible mechanisms involved in the inactivation of invertase by oxidants are discussed.

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# THE STRUCTURE OF THE COLLODION MEMBRANE AND ITS ELECTRICAL BEHAVIOR

## III. THE BASE EXCHANGE PROPERTIES OF COLLODION

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### I

In preceding papers<sup>1-3</sup> it was shown that electrochemical behavior of collodion membranes (at least in solutions of strong, weakly adsorbable electrolytes) is due to the presence of acidic impurities in the collodion. Highly purified preparations exhibit only low electrochemical activity (as measured by concentration potentials, anomalous osmosis, etc.), and in our opinion, even this low activity is due to traces of acidic impurities; the ideal pure nitrocellulose should, in solutions of strong, weakly adsorbable electrolytes, be void of any distinct electrochemical activity. It was further pointed out that the charge density at the collodion-solution interface is the ultimate determining factor. This general concept was actually used some time ago in some very interesting attempts to put the theory of electrochemical membrane behavior on a quantitative basis (Teorell,<sup>4</sup> Meyer and Sievers<sup>5</sup>).

We came to the conclusion that a study of the base exchange capacity of the collodion-solution interfaces would be the best method of obtaining pertinent quantitative data to test these theories. The base exchange capacity of a given substance is defined as the maximum cation exchange which can take place between a base exchange body and an electrolyte solution. The base exchange capacity of a given interface should be the limiting value of the possible charge density. The actual effective charge density would be the product of the base exchange capacity and the degree of dissociation (or a similar function).

In the present paper we shall attempt to determine whether or not a quanti-

<sup>1</sup> Sollner, K., and Abrams, I., *J. Gen. Physiol.*, 1940, **24**, 1.

<sup>2</sup> Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **24**, 467.

<sup>3</sup> Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **25**, 7.

<sup>4</sup> Teorell, R., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 282.

<sup>5</sup> Meyer, K. H., and Sievers, J.-F., *Helv. Chim. Acta*, 1936, **19**, 649; Meyer, K. H., *Tr. Faraday Soc.*, 1937, **33**, 1073.

tative correlation exists between base exchange capacity of collodion and its electrochemical "activity" as defined in previous papers.<sup>2,3</sup> A quantitative evaluation of these and other data and an attempt to correlate them to the above mentioned quantitative theories will be the subject of a subsequent publication.

## II

Little attention has previously been paid to this problem. Beutner, Caplan, and Loehr<sup>4</sup> carried out experiments with "Celloidin" (Schering), a brand of collodion which exhibits unusually high electrochemical activity.<sup>1-3</sup> They measured the titratable acidity of distilled water and 0.1 M potassium chloride solution into which equal numbers of cubes cut from commercial "Celloidin" gel had been placed and shaken for 48 hours. They found that both the water and potassium chloride solution became acid. The titratable acidity after this period of shaking with collodion in the case of the water was in most cases two thirds as great as with the potassium chloride solution. These workers also roughly estimated the pH of the same solutions by indicator methods and concluded that the electrolyte solution became more acid than did the distilled water; the former had a pH of about 3.8, and the latter, 4.8. Beutner, Caplan, and Loehr conclude that this differential effect determines the electrochemical activity of collodion.

Wilbrandt,<sup>7</sup> repeating the experiments of Beutner, Caplan, and Loehr, could confirm the acidulation. He attributes it to the presence of nitric acid due to the hydrolysis of the collodion. However, Wilbrandt did not perform any quantitative measurements; moreover, his analytical method is open to criticism, the diphenylamine test used not being specific for nitric acid.

Meyer and Sievers,<sup>8</sup> accepting the results of Beutner, Caplan, and Loehr, quote them in corroboration of their theory.

In addition, several studies on the base exchange properties of cellulose appear in the technical literature.<sup>9</sup> A discussion of this material, however, is outside the scope of this paper, considering the restricted bearing upon our problem. The differences between cellulose and nitrocellulose are too great to allow a ready comparison.

## III

Base exchange experiments are usually carried out in the following manner. First the material to be investigated for its base exchange properties is brought into as well defined a chemical state as possible by saturating it with cations

<sup>4</sup> Beutner, R., Caplan, M., and Loehr, W. M., *J. Biol. Chem.*, 1933, **101**, 391.

<sup>7</sup> Wilbrandt, W., *J. Gen. Physiol.*, 1935, **18**, 933.

<sup>8</sup> Meyer, K. H., and Sievers, J.-F., *Helv. Chim. Acta*, 1936, **19**, 665.

<sup>9</sup> See, e.g., Schwalbe, C. G., and Becker, E., *Ber. chem. Ges.*, 1921, **54**, 545.

of a single kind. This can be done in different ways. The sample may be treated with an electrolyte solution, *e.g.*, sodium chloride, until all ions capable of exchange are replaced by sodium. Usually it is preferable to use a solution of acid (hydrochloric acid), as in this case the necessary subsequent washing does not cause any complications due to hydrolysis which occur if salts are used. Electrodialysis also causes replacement of all other cations by hydrogen ions. We shall discuss solely hydrogen ion saturated material.

Known quantities of such material are brought into contact with a neutral salt solution. The hydrogen ions from the exchange body are replaced by the other cations in solution and the latter becomes acid. The quantity of titratable hydrogen ion in the supernatant solution represents the base exchange when no complicating reactions occur. It can be measured either by direct titration or by determining the hydrogen ion concentration with some physical method, *e.g.*, the glass electrode. The two results should check within the limits of experimental error. If necessary one takes into account such correction factors as the carbon dioxide present and the activity (degree of dissociation) of the electrolyte solutions. Any sizable disagreement between the results of the two methods would indicate definitely that one is not dealing solely with true base exchange. The treatment with salt solution is, if necessary, repeated until (practically) complete exchange is achieved. Often a single treatment is sufficient since the salt is usually present in great excess so that the hydrogen ion is replaced nearly completely, even by weakly adsorbable cations.

If shaken with pure water, an ideal base exchange body saturated with hydrogen ions does not react at all. If it behaves differently, one is not dealing with a base exchange phenomenon but with something different, most likely solubility.

Base exchange is a very fast process provided the surfaces to be tested are easily accessible. If inner surfaces of the exchange body are accessible only with difficulty or if a diffusion through the solid substance of the base exchange body makes itself felt, the process may require a long time for completion. Attention should be called to the fact that collodion membranes show their characteristic electrochemical properties practically instantaneously on contact with salt solutions.

It is obvious from the data quoted earlier that the conditions of the experiments of Beutner, Caplan, and Loehr<sup>6</sup> did not permit measurements of the true base exchange capacity of collodion. This is apparent from (1) the occurrence of large titration values for water which was in contact with their collodion and (2) the disagreement between the titration and pH values. The calculated pH values for the experiments quoted above are about 3.0 for the potassium chloride solution and 3.1 for the water. This compares with experimental values of 3.8 for the potassium chloride solution and 4.8 for water reported for a similar experiment by the same authors (see above).

## IV

In approaching our experimental problem, we first repeated the experiments of Beutner, Caplan, and Loehr using Schering-Kahlbaum "Celloidin" "for general use." We obtained experimental data very similar to theirs. (Our experimental figures are given below as cases 7 *a* and 7 *b* in Table II.)

For a more thorough investigation, it was necessary to study a great variety of collodion preparations of different origin and varying electrochemical activity. We have investigated several American and some foreign brands of collodion in the original state and after purification, and in addition, oxidized ("activated") collodion<sup>2</sup> and crude nitrocellulose. The behavior of these materials was studied before and after special purification processes. In order to obtain data which can be compared intelligently, it was necessary to study the different collodion preparations in as nearly the same physical and chemical state as possible.

Uniformity of the physical state was achieved by allowing dilute collodion solutions to drip, under standardized experimental conditions, into a great excess of vigorously stirred distilled water in which it is precipitated immediately. The fibrous products so obtained were washed superficially and dried. The precipitated material, of course, shows certain differences as to fiber length, aggregation of fibers, etc., according to the different molecular weights of the different preparations.<sup>2,3</sup> The material precipitated from solution gives a large surface for possible base exchange, certainly not smaller than the same weight of collodion in the form of membranes.

The ideal chemical state would require that all the acid groups in the collodion should be present in the free acid state, *i.e.*, saturated with hydrogen ions. The extent to which this condition is fulfilled with our different collodion preparations is difficult to state exactly. In their manufacture they certainly have undergone a most thorough acid treatment. Nevertheless, they all contain some ash, though mostly in rather minute amounts. This ash is not composed of basic material only; it always contains some sulfate which was introduced in the manufacturing process. In the collodion, the sulfate is probably present partially in the semi-esterified form, partially in the free, uncombined state, the latter form being prevalent in crude (not thoroughly purified) collodion. If present as an acid ester, it contributes to the true acidity of the collodion; the uncombined fraction is present as mechanically admixed impurity, partially or entirely combined with some of the bases. A corresponding quantity of acid and base would thus be neutralized, neutralized also in their effect on the apparent base exchange properties of the collodion. It is very difficult to remove the last traces of these impurities, but, as shown previously,<sup>2</sup> a large fraction can be extracted without seriously affecting the electrochemical activity of the collodion. One must also consider the fact that the impurities are distributed throughout the mass of the collodion; only a small fraction

could lie in the interfaces, where they might affect the electrochemical behavior. Moreover, water soluble, not strongly adsorbable impurities, *e.g.*, inorganic ions can readily be washed off the collodion surfaces by treatment with water. The possible error in our base exchange experiments which could be due to the ash and sulfate content is insignificant, as will become apparent.

It is important to emphasize here that our exchange studies were carried out not with the native or purified fibers, but with freshly prepared fibrous material, obtained by dissolving our preparations in ether-alcohol and precipitating in distilled water. This was done with all samples unless otherwise indicated. This procedure assures one that only surfaces corresponding in their composition to the properties of mass of the collodion are studied. Without it, the experiments would be subject to the objection that surfaces were studied which had undergone a special treatment and thus are not representative of the collodion. With membranes, of course, one is always dealing with surfaces originating from dissolved material. The collodion surfaces used for the base exchange studies were, in this respect, analogous to those which occur in membranes cast from the same collodion solutions.

The exchange properties of the precipitated fibers were found to be the same, whether used with or without drying. For the sake of convenience, we used dried<sup>10</sup> material in most cases.

The *commercial collodion preparations* which we used in this work were precipitated from ether-alcohol solutions without any special purifying operations.

Most of the *preparations which we purified* ourselves were treated with 60 per cent acetic acid or alcohol-water mixtures, as indicated in Table II, column 1. Great care was always taken to prevent the uptake of cations by using double distilled water.

The "*oxidized*" collodion was prepared from a pure commercial preparation by immersion for 24 hours in a sodium hypobromite solution, prepared by adding to molar sodium hydroxide solution enough bromine to adjust the pH to about 7 as described previously.<sup>8</sup> The oxidized product was washed with water, dilute hydrochloric acid, and again, very thoroughly with double distilled water. The ash (and sulfate) content of properly purified oxidized collodion is very low, lower than that of the original material. Low molecular weight decomposition products originating from the oxidation process are also removed by the indicated treatment. For some of our experiments, this oxidized collodion was further purified by repeated washings with aqueous alcohol. We may add here that oxidized collodion prepared by immersion in sodium hydroxide<sup>8</sup> behaves substantially the same as collodion oxidized with hypobromite. However, it is somewhat more difficult to free of low molecular

<sup>10</sup> That dried collodion must be handled with due care was pointed out previously (Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, 25, 7).

weight decomposition products on account of the stronger degradation associated with the alkaline treatment.

The samples of *crude nitrocellulose* listed in the tables, as well as several others which are not listed, were obtained through the courtesy of the Monsanto Chemical Company, Plastics Division.<sup>11</sup> According to the information received, they were taken from the manufacturing process at the end of the washing to eliminate free acid, but before the purification steps intended to remove sulfuric esters and other impurities. The samples of crude material listed in Tables I and II are representative for crude collodion; several other lots which we have tested had very similar properties. Sample A was received wet with alcohol, having been washed several times with 95 per cent alcohol. Sample B was obtained in the water wet state. The combined effect of proper purification operations, such as boiling with water and boiling or repeated washing with alcohol of suitable concentration, allows one to prepare from sample B a product which is practically identical in its properties with sample A. In the same way, any desired degree of purity, base exchange capacity, and activity can be obtained with material from either batch at will. Several samples treated in the described manner are listed in Table II. Of course, each batch of crude collodion is slightly different as to ash and sulfate content, base exchange properties, and activity, according to the moment at which it was withdrawn from the regular manufacturing process.

V

Since the experiments of Beutner, Caplan, and Loehr<sup>6</sup> indicate that at least some collodions do not show simple, true base exchange, each preparation was studied not only with an electrolyte solution but also with distilled water.

The electrolyte chosen was 0.5 M potassium chloride after we had established the fact that the electrolyte concentration is rather unimportant, provided it is not too low. All water used was double distilled and reasonably free from carbon dioxide (pH > 6.5). The salt solutions used in all our experiments showed pH values from 6.5 to 7 before the experiment, as measured with a Leeds and Northrup glass electrode.

All experiments were carried out in Pyrex Erlenmeyer flasks with glass stoppers which were properly steamed and aged before use. Water and potassium chloride

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<sup>11</sup> We are greatly indebted to the Monsanto Chemical Company for these samples, particularly to Mr. H. K. Nason, Assistant Director of Research, to whom we would also like to express our special thanks for very valuable information on crude nitrocellulose.

Since we have a large sample of crude water-wet nitrocellulose on hand, we shall be glad to place samples of this material at the disposal of other interested investigators as long as our supply remains.

solutions kept in them for several days did not show any detectable change in pH (6.5 to 7 before and 6.5 to 7 after).

The titration values given in Tables I and II are milliliters of 0.01 *N* sodium hydroxide solution per gram of dry collodion. The titrations were carried out with a microburette, phenol red being used as indicator. The accuracy is about 0.03 ml. 0.01 *M* sodium hydroxide solution. The pH determinations may be assumed to be accurate to better than 0.1 unit.

## VI

As indicated above, the influence of the time factor on the base exchange must be known. Table I gives titration and pH values obtained with two "active" collodion preparations at several reaction times.

Oxidized collodion was prepared by treating U.S.P. Baker Collodion Cotton with "molar" sodium hypobromite solution for 24 hours, as described above, followed by thorough washing. The "crude nitrocellulose" was prepared from sample "B" by removal of excess free sulfuric acid by boiling with water for several hours. Both samples were precipitated as described above.

9 gm. of dry collodion were added to 300 ml. of water and potassium chloride solution. These samples were shaken for several minutes by hand and allowed to react further under occasional shaking. 10 ml. samples of the clear supernatant fluid were removed after measured periods and their pH determined with the glass electrode. The samples were then titrated with sodium hydroxide solution.

Table I shows that base exchange with precipitated collodion is a rather slow process. Inaccessible inner surfaces or diffusion processes in and out of the solid material, or both, obviously play an important rôle. A material with an open structure, particularly if in a finely divided state, as is the case with our precipitated fibers, should reach base exchange equilibrium within very short periods.

An appreciable difference between the two preparations investigated is apparent. The precipitated crude collodion obviously has a more open structure than the precipitated oxidized collodion. The latter, though it eventually yields a greater base exchange, required about 6 hours to exchange to the same extent as the crude nitrocellulose in 15 minutes. The microscopic differences between the two preparations were too small to account for this difference. We are thus forced to conclude that the precipitated fibers have a somewhat different submicroscopic structure which obviously must result from molecular differences.

Table I also shows that the two tested collodion preparations fulfill, at least very closely, the first of the conditions necessary for true base exchange, namely, no reaction with water, which could be considered to be of real significance. The probable meaning of the slight shift of pH in the water will be discussed



briefly below. There we will also see that the other necessary condition for true base exchange is also fulfilled, namely, agreement between the acidity values as found with our two independent methods, titration and electrometric pH determination.

Following the example of Beutner, Caplan, and Loehr,<sup>6</sup> we arbitrarily chose 2 days as the standard reaction time for our further experiments, though

TABLE I

*The Influence of the Time of Reaction on the Base Exchange of Active Collodion Preparations*

A. Oxidized collodion				
Time of reaction	Ml. 0.01 N NaOH per gm. dry collodion on treatment with		pH values on treatment with	
	0.5 M KCl	Water	0.5 M KCl	Water
15 min.	0.00	0.00	5.6	6.6
1 hr.	0.00	0.00	5.3	6.6
6 hrs.	0.05	0.00	4.8	6.6
24 hrs.	0.13	0.00	4.5	6.3
2 days	0.29	0.00	4.1	6.3
1 wk.	0.54	0.00	3.9	5.9
2 wks.	0.57	0.00	3.9	5.9
5 wks.	0.56	0.00	3.9	5.6
B. Crude collodion				
15 min.	0.05	0.00	4.8	6.7
1 hr.	0.13	0.00	4.5	6.7
6 hrs.	0.20	0.00	4.3	6.4
24 hrs.	0.27	0.00	4.2	6.4
2 days	0.30	0.00	4.1	6.3
1 wk.	0.35	0.00	4.1	6.0
2 wks.	0.39	0.00	4.1	5.8
5 wks.	0.39	0.00	4.1	6.4

much could be said in favor of a shorter time. However, the selection of the 2 day period is bound to bring out more clearly the differences between the active and inactive preparations. It has the further advantage of increasing the absolute effects, thus rendering them easier to determine.

The arbitrary choice of the reaction time, whatever its length may be, necessitates a brief discussion concerning the exact meaning of the experimental data. We measure the actual base exchange which takes place during the standard time. Table I indicates—and this is of importance—that our measurements listed in Table II give values much greater than those which in all probability correspond to the dissociable groups which cause the characteristic electrochemical behavior of membranes. The latter, as we may repeat here, appears

nearly instantaneously on contact with electrolyte solutions. All the values, therefore, obtained with our standard method are decidedly higher than those which should be correlated to the electrochemical activity of collodion. We will have to consider the consequence of this fact later.

It may be noted that repeated renewal of the electrolyte solution does not alter the measured base exchange in a given period of time, as compared with that obtained when the collodion is in contact with the same solution over the whole period.

## VII

In Table II are listed the base exchange and activity data obtained with some representative collodion preparations of different origin and different pretreatment. In all cases, unless indicated otherwise, precipitated fibers were used.

50 ml. of water or 0.5 M potassium chloride solution were added to 1.5 gm. of dry collodion in 125 ml. Pyrex Erlenmeyer flasks. In the case of the wet preparations, an amount of collodion corresponding to 1.5 gm. of dry material was taken. The samples were shaken for several minutes and allowed to react for 48 hours with occasional shaking. After this, 10 ml. samples of the supernatant, clear, fiber-free liquid were titrated; in another sample, the pH was determined with a glass electrode. Column 2 of Table II gives the brand and pretreatment—if any—of the studied collodion preparations. In the cases of "Celloidin Schering-Kahlbaum cut in cubes" (No. 7 *a* and 7 *b*)—following the procedure of Beutner, Caplan, and Loehr—the stiff (about 20 per cent) commercial Celloidin alco-gel was cut in small cubes (about  $4 \times 4 \times 3$  mm.) and treated exactly as the other (fibrous) preparations. Here too, the listed values are per gram of dry substance.

In columns 3 and 4 are listed the ash and sulfate content of our preparations, determined as described previously.<sup>2</sup>

Columns 5 and 6 indicate the milliliters of 0.01 N NaOH solution used per gram of dry collodion after 48 hours contact with 0.5 M KCl solution and with water respectively.

Columns 7 and 8 show the pH values (as determined with a glass electrode) of the KCl solution and water after 48 hours contact with collodion. Whether or not any conclusions can be drawn from the experimentally measured pH values which lie between 5.3 and 7 will be discussed below.

Columns 9 and 10 give the pH values calculated from the titration values of columns 5 and 6 under the assumption that the NaOH used has neutralized HCl in an unbuffered system.

On account of the experimental limitations of our titrations, titration values of 0.00 ml. 0.01 M sodium hydroxide correspond in our table to calculated pH values which may lie anywhere between 5 and 7. Therefore, the calculated pH values in columns 9 and 10 are given in all these cases as  $>5$  to indicate the limitations of our volumetric experiments.

Columns 11 and 12 indicate the electrochemical activity of the different collodion

preparations. Bag-shaped membranes were prepared as described previously.<sup>1,3</sup> The same method of characterization was used as in preceding papers.<sup>2,3</sup> The membranes tested were as far as possible of the same porosity, as indicated by their behavior when tested with sucrose solution. The figures in column 11 indicate the millimeter pressure rise observed 20 minutes after the membranes filled with 0.25 M sucrose solution were placed in distilled water. Column 12 gives the anomalous osmotic rise in millimeters of water obtained after 20 minutes with  $\frac{M}{512}$  potassium sulfate solution. As shown previously,<sup>2,3</sup> the rate of this rise is a rather sensitive indicator of the electrochemical activity of collodion.

### VIII

Before discussing the main results of Table II, it is necessary to clarify some technical points. First, we may consider the question of the importance of the ash and sulfate content of our collodion preparations. As was pointed out above, there is no indication that the ash and sulfate content should appreciably influence the base exchange (and activity) of collodion. This view is substantiated by a close inspection and comparison of the data of columns 3 and 4, 5 and 6, 7 and 8, (and 12) of Table II; particular attention should be paid to the data referring to the genetically related samples 7, 7a, 8, and 9; 10 and 10b; 11 to 14. High ash and sulfate content is compatible with zero base exchange and high pH values (see particularly cases 6, 8, 9, 10, and 14). *In the process of precipitation the surfaces of collodion have obviously been freed thoroughly from soluble impurities by their contact with water, though some of the sulfate may be present in an insoluble combined form. The bulk of the sulfate and ash found by analysis is obviously so thoroughly enclosed in the fibers that no significant amount is able to diffuse out within short periods. These impurities, therefore, can be disregarded completely for our further discussion.*

The fact that drying does not seem to affect the base exchange properties of our precipitated fibers can be concluded from a comparison of cases 10 and 10a, 12 and 12a.

Case 10b, crude collodion in the state of native fibers (nitrated cotton) which was obtained in the alcohol wet state, is quoted to give an idea of the characteristics of such preparations.

Let us next consider the data obtained with the same material as that used by Beutner, Caplan, and Loehr<sup>4</sup>—"Celloidin." Cases 7a and 7b are as accurate repetitions as possible of the experiments of these authors. The results listed in columns 5 to 8 are in complete agreement with theirs. We want to emphasize here again that some substance or substances which neutralize sodium hydroxide on titration obviously were dissolved in the water. Therefore, we are not dealing with a simple base exchange. This conclusion can also be derived from the fact that there is a large discrepancy between the experi-

mentally found pH values and those calculated from the titration data. For the "base exchange" with water, such a calculation is, of course, absolutely fictitious. The pH values indicate that the dissolved substances were either very weakly acid or contained some acidic component. In the case of the "base exchange" with potassium chloride solution the situation is substantially the same, though it may be that some (though very small) true base exchange occurred. A pH of 4.5 corresponds in our experiments to a titration value of only about 0.1 ml. 0.01 M sodium hydroxide. As we are dealing with acidic impurities, a buffering action upon the hydrochloric acid which may have been developed by true base exchange seems to be ruled out. Here again we are dealing mainly if not exclusively with some process of dissolution.

The nature of the soluble substances is without particular interest here, but this much must be said. It is known that inadequately purified nitrocellulose ("Celloidin" comes into this class<sup>1,2</sup>) not only contains water-soluble material which normally is removed by purification, but also decomposes spontaneously in course of time<sup>12</sup> as shown on a large scale by Silberrad and Farmer.<sup>13</sup> Nitrous compounds are formed in such preparations. They act upon the nitrocellulose forming many kinds of lower molecular weight substances mostly of an acidic nature. Moreover, they obviously render a fraction of the nitrocellulose water-soluble. These water-soluble compounds probably account for a very great part of the high titration values with sodium hydroxide solution. It is known that alkalis do not react in a straightforward manner with many "nitro" compounds such as nitric acid esters, but destroy them in an oxidative manner with the formation of nitrites,<sup>3,14</sup> etc. 1 gm. of nitrocellulose in contact with an excess of sodium hydroxide uses a quantity which corresponds to more than 1000 ml. of 0.01 N hydroxide solution. Thus a very small amount of dissolved nitro-compound may use up large quantities of hydroxide solution.

Here the question arises as to how far the behavior of the "Celloidin" gel cut in cubes is paralleled by the base exchange properties of material prepared from solutions of this preparation, as membranes would necessarily be. Case 7 of

<sup>12</sup> Such spontaneous decomposition of "Celloidin" accounts also for the inability of Michaelis and collaborators (Green, A. A., Weech, A. A., and Michaelis, L., *J. Gen. Physiol.*, 1929, **12**, 473) to produce highly dried collodion membranes having a constant electrical conductivity, as suggested already by the above authors. One can expect that membranes prepared from better stabilized collodion would not present the same difficulties.

<sup>13</sup> Silberrad, O., and Farmer, R. C., *J. Chem. Soc.*, London, 1906, **89**, 1182. For further references, see particularly Schwalbe, C. G., *Die Chemie der Cellulose*, Berlin, Borntraeger, 1910-11 and 1918, 289.

<sup>14</sup> Will, W., *Ber. chem. Ges.*, 1891, **24**, 400; Silberrad, O., and Farmer, R. C., *J. Chem. Soc.*, London, 1906, **89**, 1759. For further references, see particularly Schwalbe, C. G., *Die Chemie der Cellulose*, Berlin, Borntraeger, 1910-11 and 1918, 293.

TABLE II  
Base Exchange and Activity of Various Collodion Preparations

1	2	3	4	5	6	7	8	9	10	11	12
	Brand of collodion and pretreatment (All preparations were precipitated from ether-alcohol solutions and dried, unless stated otherwise)	Ash and SO <sub>4</sub> content		Base exchange data						Electrochemical activity	
		mg. ash per gm. dry collodion	mg. SO <sub>4</sub> per gm. dry collodion	Ml. 0.01 N NaOH per gm. dry collodion on treatment with		pH values found experimentally on treatment with		pH values calculated from titration values of columns 5 and 6		Osmotic rise with 0.25 M sucrose	Anomalous osmotic rise with 5% K <sub>2</sub> SO <sub>4</sub>
				0.5 M KCl	Water	0.5 M KCl	Water	0.5 M KCl	Water		
										mm.	mm.
1	Mallinckrodt "Parlodion," commercial preparation	0.16	0.04	0.00	0.00	5.4	6.2	>5	>5	115	40
										124	43
										130	35
2	Mallinckrodt "Parlodion," boiled 15 hrs. in alcohol	Very low	Very low	0.00	0.00	5.9	5.9	>5	>5	106	15
										128	16
										136	28
3	Merck collodion U.S.P., commercial preparation	0.25	0.2	0.00	0.00	5.6	5.8	>5	>5	116	48
										125	46
										138	57
4	Baker collodion U.S.P., commercial preparation	0.45	0.2	0.00	0.00	5.3	5.9	>5	>5	126	50
										128	32
										130	47
5	Schering-Kahlbaum collodion "zur Herstellung von Membranen," commercial preparation	1.3	0.9	0.13	0.06	5.1	5.7	4.4	4.7	118	98
										122	89
										130	104
6	Schering-Kahlbaum collodion "zur Herstellung von Membranen," boiled 8 hrs. in 60 per cent acetic acid	1.0	0.4	0.00	0.00	5.4	6.5	>5	>5	118	95
										126	102
										152	134
7	Schering-Kahlbaum "Celloidin" "for general use," commercial preparation	3.6	2.0	0.06	0.00	5.2	5.8	4.7	5	110	190
				0.27	0.00	5.1	5.8	4.1	5	120	180
										126	228
7a	Same as No. 7, but not precipitated, the commercial gel cut in cubes	3.6	2.0	3.74	1.89	4.7	5.0	2.95(1)	3.2(1)		
7b	Similar to No. 7a, but aged several months in the laboratory			1.05	1.26	4.5	4.5	3.5(1)	3.4(1)		
8	Schering-Kahlbaum "Celloidin" "for general use," boiled 3 hrs. in 60 per cent acetic acid	1.2	0.9	0.00	0.00	5.1	6.3	>5	>5	116	132
										128	135
										136	150
9	Schering-Kahlbaum "Celloidin" "for general use," boiled 6 hrs. in 60 per cent acetic acid	1.0	0.4	0.00	0.00	5.2	6.5	>5	>5	131	143
										138	138
										145	156

Table II shows the behavior of "Celloidin" after precipitation from ether-alcohol solution in water. There is no "base exchange" with water while with the potassium chloride solution, there is some appreciable reaction. Obviously, most of the soluble and reactive material is removed from the surfaces of the precipitated fibers in the very process of precipitation; no appreciable diffusion of such material from the interior of the fibers takes place, though the salt solution is obviously able to peptize some material (which reacts on titration with sodium hydroxide) as indicated by the discrepancy between the values in columns 7 and 9.

The large differences in the "base exchange" properties of "Celloidin" gel

TABLE II—*Concluded*

1	2	3	4	5	6	7	8	9	10	11	12
10	Crude collodion cotton sample A (which was obtained washed with alcohol)	1.1	0.8	0.00	0.00	5.7	5.8	>5	>5	105 115 115	150 160 168
10a	Crude collodion cotton sample A, not dried after precipitation			0.00	0.00	5.6	5.9	>5	>5		
10b	Crude collodion cotton sample A in the original fibrous state, (not precipitated)*	2.3	2.8	1.40	1.27	3.5	3.6	3.4	3.4		
				2.02	1.40	3.3	3.5	3.2	3.4		
11	Crude collodion cotton sample B*	1.0	3.4	0.67	0.00	3.9	5.0	3.7	>5	122 136 154	146 150 182
12	Crude collodion cotton sample B, boiled 3 times 3 hrs. each with water	0.5	0.7	0.26	0.00	4.1	5.6	4.1	>5	120 128 135	126 133 147
12a	Same as No. 12, but not dried after precipitation			0.23	0.00	4.1	5.8	4.1	>5		
13	Crude collodion cotton sample B, washed 6 times with 95 per cent alcohol and boiled 3 times 30 min. each with water	1.0	1.4	0.06	0.00	4.7	6.1	4.7	>5	120 136 142	146 152 180
14	Crude collodion cotton sample B, boiled 7 hrs. with 70 per cent alcohol (2 alcohol changes)	1.6	1.0	0.00	0.00	5.8	6.6	>5	>5	112 136 140	133 148 154
15	Oxidized collodion; (Baker collodion cotton, "Pyroxilin") oxidized 24 hrs. with 1 M NaOBr	0.3	<0.2	0.27	0.00	4.2	6.4	4.1	>5	118 130 137	130 142 148
16	Oxidized collodion (No. 15), washed 8 times with 95 per cent alcohol	<0.3	<0.2	0.13	0.00	4.5	6.2	4.4	>5	124 127 132	120 122 130
17	Oxidized collodion (No. 15), washed 16 times with 95 per cent alcohol	<0.3	<0.2	0.00	0.00	5.0	6.7	>5	>5	132 137 140	119 128 131
18	Oxidized collodion (No. 15), washed 25 times with 95 per cent alcohol	<0.3	<0.2	0.00	0.00	5.3	6.5	>5	>5	120 125 139	92 104 118

\* This preparation contains much free sulfuric acid.

and fibers prepared from the same material make it improper to transfer data obtained with the former to the problem of the electrochemical behavior of membranes.

Precipitated Schering-Kahlbaum Collodion "zur Herstellung von Membranen" (case 5 in Table II) gives off small quantities of soluble material even into water, but is otherwise similar to the precipitated "Celloidin" (case 7). Like Schering-Kahlbaum "Celloidin", Schering-Kahlbaum Collodion "zur Herstellung von Membranen" was also classified in previous reports<sup>1, 2</sup> as a poorly purified product.

## IX

It is obvious that the collodion preparations mentioned in the preceding paragraphs are unsuitable for our purpose, namely, the study of the true base exchange properties. Only preparations which give zero titration values with water, and in addition, a reasonably good agreement between the experi-

mentally found pH values of column 7 and the calculated values of column 9 are suitable for this purpose. Approximate agreement between the two assures one that true base exchange occurs, though some other factor (solubility) may make itself felt to some extent. Fortunately, proper purification yields products of the desired properties.

Agreement ordinarily within the limits of our experimental (titration) error exists in cases of the precipitated preparations, 1, 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, and 15 to 18. None of these preparations, it may be noted, shows a reaction with water sufficiently large to be detected by titration. Cases 11, 12, 13, 15, and 16, precipitated crude collodion and precipitated oxidized collodion, show measurable base exchange values. In all the other cases, (1, 2, 3, 4, 6, 8, 9, 10, 14, 17, 18), the base exchange is too low to be measurable by titration (*i.e.*, less than 0.03 ml.), and correspondingly, the pH values are greater than 5.

Focusing our attention now entirely on the cases mentioned in the preceding paragraph, we may finally turn toward our main objective, namely, the possible correlation of base exchange properties and electrochemical activity (as exemplified by the data on anomalous osmosis—column 12 of Table II) of the different samples of precipitated collodion.

If we take the titration values as the measure of the base exchange we find (1) cases which combine “zero” base exchange with low electrochemical activity, No. 1, 2, 3, and 4; (2) cases which combine measurable base exchange with great activity, No. 11, 12, 13, 15, and 16; (3) cases which combine “zero” base exchange with great activity, No. 6, 8, 9, 10, 14, 17, and 18.

As yet, we have not found a case combining chemically measurable base exchange with low activity.

It is particularly interesting to compare the genetically related samples: 5 with 6; 7 with 8 and 9; 11 and 12 with 13 and 14; 15 with 16 and 17 and 18. The relatively small, sometimes hardly significant changes in activity are somewhat in contrast with the large differences in base exchange.

*The general result of Table II can thus be summarized by saying that no regular correlation exists between chemically measurable base exchange capacity and electrochemical activity.*

Low as well as high activity can be associated with a base exchange capacity too low to be measurable by titration. High, *i.e.*, measurable base exchange capacity always seems to be correlated with high electrochemical activity.

If we turn our attention now to the correlation between pH values and electrochemical activity, we arrive at exactly the same classification as before if we arbitrarily take pH 5 as a demarcation line. This, of course, is simply a necessary consequence of the fact that in all the cases which are considered here, a reasonable quantitative agreement exists between pH and base exchange data.

But now the question arises as to how far pH values greater than 5 may be

used as a basis for more far-reaching conclusions. It is not possible to answer this question without discussing some possible errors and the limitations of the method. Small pH changes near the neutral point, of course, mean very little if taken singly; but consistently analogous data when taken as a whole may have considerable weight and may be used for certain limited conclusions.

If our collodion preparations were perfectly inert substances, water in contact with them should not undergo a change in pH. However, this is not the case. The pH on treatment with water varied between 5.7 and 6.7 for the different samples tested.<sup>15</sup> It is somewhat paradoxical that some of the "inactive" pure commercial preparations caused a greater shift in pH than most of the purified "active" samples. This is easily understood, however, on the basis of the fact that even very pure collodion is not absolutely stable. The "inactive" preparations had stood for some time before use and thus contained more soluble acidic impurities than the recently purified "active" preparations. This agrees also with the fact that some experiments, not listed in the tables, demonstrate that our specially purified products cause a greater shift in pH if not used in the freshly prepared state.<sup>16</sup>

Since solubility effects may change the pH even in pure water from neutrality (pH 6.5 to 7) to a pH of 5.7, we are inclined to believe that such effects are appreciably stronger in salt solutions as the latter may have some peptizing effect. Without a special and thorough investigation of each individual case, it is impossible to say with accuracy how great a fraction of the pH change observed in potassium chloride solution must be attributed to true base exchange and how large a proportion to solubility. In some cases, e.g., in the not specially purified samples 1 and 4, the latter fraction is probably rather large. In cases 8, 17, and 18, on the other hand, true base exchange is most likely the prevailing factor, as can be concluded from a comparison with the respective genetically related samples.

No matter what limiting value we assume for the significance of the pH measurements in the potassium chloride solutions, i.e., whether 5.3 or 5.6, the main result of our investigation remains quite the same, though one or two cases (No. 1 and No. 4) would now be listed under a fourth heading, namely, cases which combine base exchange measurable by physical means with low electrochemical activity. *With the present methods, no regular correlation can be found empirically between base exchange capacity as determined by physical methods and electrochemical activity.*

<sup>15</sup> Here we must disregard case 11 which is obviously influenced by its high content of free sulfuric acid, traces of which diffuse into the water during the long—48 hours—contact time.

<sup>16</sup> We have gained the impression that extremely well purified collodion preparations also show a slight increase in activity when stored for prolonged periods; this is obviously likewise due to a slow decomposition and oxidation.



From the point of view of a membrane investigation, a more thorough study of the factors mentioned in the last few paragraphs would become a pertinent problem only after it has been shown that such a study could contribute materially to our fundamental knowledge of membrane structure. A more detailed discussion of any possible quantitative correlation of base exchange capacity and electrochemical activity would necessarily have to wait for much more accurate data. But for the reasons discussed above, it may be very difficult, perhaps impossible, to obtain such data with collodion. However, at this point, the following statement can be made with certainty: *The base exchange capacity which is necessary to cause even great electrochemical activity of collodion is very small, just at the borderline of usefulness of the present experimental technique, more probably even beyond it.*

We have arrived at this result without taking into account the time effect (Table I). Here we must remember that the electrochemical properties of collodion membranes are apparent nearly instantaneously on contact with electrolyte solutions. This means that the electrochemical behavior of membranes obviously is determined by those ionizable groups which lie exposed in the water-solution interface in the pores. It therefore would be preferable in a more thorough discussion of the correlation of electrochemical activity and base exchange to consider the base exchanges after short periods. Thus, the base exchanges which should be taken as the basis for any discussion are actually much smaller than the values of Table II, which refer to the exchange situation in fibrous collodion after 48 hours.<sup>17</sup> Therefore, the italicized statement made above concerning the magnitude of the base exchange which is necessary to produce great electrochemical activity is true in an even more pointed manner. *The base exchange which corresponds to the true physical cause of the electrochemical activity of collodion is much too small to be determined with the available methods.* Considering the instability of collodion, we doubt whether even a great improvement in technique could lead with this material to significant results.

In spite of this somewhat puzzling result reached in the last paragraph, there is no escape from the conclusion reached previously<sup>1, 2</sup> that dissociable groups built into the collodion cause its electrochemical activity. Preferential ion adsorption was shown to be ruled out as a major factor, at least in solutions of strong, weakly adsorbable ions. Such an assumption could never explain the differences in electrochemical activity which are observed with different collodion preparations.

A visualization of the true situation can obviously be found in the direction

<sup>17</sup> On the basis of these considerations, one might expect that membranes prepared from the crude collodion (referred to in Table I b) would have at least not a lower electrochemical activity than those prepared from the oxidized collodion (Table I a). This is actually the case as can be seen from No. 12 and No. 15, columns 11 and 12 in Table II, which refer to the same preparations as Table I a and I b.

of the following reasoning: measurable base exchange disappears earlier on purification than the pronounced electrochemical activity. Our methods of determining the two are of different sensitivity. The electrochemical properties of the collodion, *i.e.*, of the collodion-solution interfaces, are determined by a smaller number of ions than can be detected with certainty in the external solution. By proper and prolonged purification, one can reduce the number of dissociable groups contained in collodion to a very low level. Finally even the electrochemical activity disappears nearly entirely, as can be seen from the low "activity" of highly purified collodion. Cases 1, 3, and 4 refer to commercial collodion preparations. No. 1 (Mallinckrodt "Parlodion"), which is manufactured for greatest purity, is the purest and least active among all the many commercially available preparations we have tested. Case 2 is of particular interest since it shows that even this specially purified material loses activity when further purified. For this, fibrous Mallinckrodt "Parlodion" was boiled for 15 hours with 90 per cent alcohol (about 18 gm. of material per liter alcohol); the latter was changed twice to remove more thoroughly the soluble material. There is no reason to believe that even the very low residual activity of the purified "Parlodion" could not be reduced further by proper methods. Any activity above this level is therefore due to impurities foreign to the ideal nitrocellulose. In fact, we can be certain that the true reference point of activity, which is caused by the presence of dissociable impurities, lies considerably below the level obtained with the purified "Parlodion." We may even venture to speculate that pure ideal nitrocellulose should be nearly completely void of any distinct electrochemical properties when immersed in pure water or aqueous solutions of strong inorganic electrolytes. Of course, at this low level of electrochemical activity, preferential ion adsorption could become an important factor even with weakly adsorbable electrolytes.

#### X

The bearing of the results of our base exchange studies upon the general problem of electrochemical membrane behavior is obvious, at least in a qualitative way.

It seems difficult to reconcile our results with the homogeneous phase theory on account of the extremely small concentration of active groups, which in some way or the other, obviously determine the electrochemical behavior of membranes. On the other hand, the pore theory too seems to run into great difficulties, at least when assuming more or less uniform, prismatic pores, as is tacitly assumed in the proposed mathematical theories. We should like to indicate briefly a line of reasoning which may overcome these difficulties, namely, the assumption of a geometrical-structural irregularity, such as must necessarily be assumed, *a priori*, for a microheterogeneous system such as the collodion membrane. If we think of pores not as uniform prismatic channels

but as random sequences of narrow constrictions and wide cavities, it is immediately evident that the narrowest constriction in each possible channel determines the permeability of the latter. A single charge at a point of constriction could easily determine the electrochemical behavior of the whole pore. Thus a very small number of dissociable groups in a membrane, less than that contained in a monomolecular film of equal dimensions, could, if properly distributed, determine the electrochemical behavior even of voluminous structures. It would be interesting to follow up some consequences of this general concept and of its implications when applied to biological systems. It likewise would be attractive to compare these ideas with certain theories of membrane structure and membrane behavior which are based on somewhat opposite assumptions.<sup>18</sup>

However, we prefer to postpone a more detailed discussion of these points to a later date, until certain studies dealing with the uptake of liquid from water and electrolyte solutions by dried collodion membranes and with their swelling will, it is anticipated, contribute data which may allow us to discuss profitably on an experimental basis the controversial problem of continuous phase theory *versus* pore theory. The true situation seems to be less simple than assumed by either theory. The above mentioned controversy obviously must be settled at least to some extent before an attempt is made to deal with a quantitative membrane theory. A quantitative test of certain aspects of the outstanding examples of an electrochemical membrane theory based on a correlation of concentration potential measurements and base exchange studies on membranes is now being carried out. Using the methods (and preparations) described in this paper, it will show clearly the *quantitative* significance of base exchange studies for the problem of electrochemical membrane behavior.

#### SUMMARY

1. Theoretical considerations lead to the conclusion that dissociable acidic groups present to a varying extent in different collodion preparations determine the electrochemical behavior of membranes cast from these preparations. It is further reasoned that the base exchange capacity of the collodion surfaces is the true quantitative measure of the abundance of the dissociable groups.

2. The concept of base exchange capacity and the base exchange method are discussed. The conditions which allow a purposeful application of the latter are stated.

3. The base exchange properties of a number of fibrous collodion preparations of different origins and after various types of treatment, having widely varying electrochemical activities, are determined.

<sup>18</sup> See, e.g., Manegold, E., *Kolloid-Z.*, 1932, **61**, 140; and many other papers by the same author in the *Kolloid-Zeitschrift*.

4. With the chemical (titration) and physical (electrometric) methods employed, no regular correlation can be found between electrochemical activity and base exchange. The base exchange capacity which is necessary to cause even great electrochemical activity of collodion is extremely small.

5. Measurable to high base exchange capacity always seems to be associated with good or high electrochemical activity; but base exchange capacity too low to be definitely measurable with the available methods may be found with collodion preparations of high as well as with preparations of low electrochemical activity.

6. The bearing of these results upon the problem of the spatial and electrical structure of the collodion membrane is indicated briefly.



# STIMULATION OF CELLS BY INTENSE FLASHES OF ULTRAVIOLET LIGHT\*

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## PLATE 5

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### INTRODUCTION

The effects of ultraviolet light, applied for considerable periods of time on a wide variety of cells and tissues, are well known.<sup>1</sup> The action is often spoken of as stimulation, since definite changes (the response) appear in the cells, just as we speak of stimulation of the skin by light when bronzing occurs, or of stimulation of the retina when impulses appear in the optic nerve.

The question arises as to whether stimulation by ultraviolet light is similar to the stimulation of a muscle or nerve, since it lacks some of the fundamental characteristics of stimulus reactions. It is not usually applied in a sudden *intense* dose and the response is not of an all-or-none character. With ultraviolet irradiation there is a definite relation between dosage and effect, which is frequently irreversible.

It is usual to think of a stimulus reaction as presenting the following well known characteristics: (1) Many kinds of *sudden* stimuli should evoke a reversible response which depends on the cell stimulated. (2) There should be no continuous relation between the energy of the stimulus and that of the response, the all-or-none effect, similar to trigger action. (3) An action potential should appear, with stimulated region electrically negative to other regions. (4) The electrical change should be propagated as a wave of negativity and decreased impedance. (5) The effect should be associated with a refractory period.

However, the last four of these categories are all phenomena connected with the propagation of the excitation process and need not necessarily appear. It is well known that *Nitella* cells do not in all respects obey the all-or-none law (Osterhout, 1936), while the work of Gelfan (1930, 1931, 1934),

\* An abstract of this paper appeared in *Biol. Bull.*, 1941, **81**, 291.

<sup>1</sup> See Duggar, B. M., Biological effects of radiation, 1936, McGraw-Hill, 1936, and Laurens, H., Physiological effects of radiations, New York, The Chemical Catalog Co., Inc., 1933.

Gelfan and Gerard (1930), Gelfan and Bishop (1932), Pratt (1930), Brown and Sichel (1936), Steiman (1937), and Ramsey and Street (1938) has shown that a single muscle fiber can respond in a graded manner to electrical stimuli of varying intensity. The graded response appears only under special conditions such as pore electrodes for stimulation, increased K in the medium, if muscle ends are cut or the muscles fatigued. No action potential appears and there is no propagated impulse. The time constants of this response are essentially the same as when a propagated excitation occurs, but there is no refractory period (Sichel and Prosser, 1940). Katz (1937), Hodgkins (1938), and Schmitt and Schmitt (1940) have also demonstrated subthreshold potentials in nerve which are graded and not propagated.

The word stimulation is so widely used for any kind of changed conditions resulting in a response that it is of interest to inquire into details of the response to ultraviolet light, particularly whether ultraviolet can ever act in the same sense as do electrical or mechanical stimuli in the excitation of a local change or a propagated disturbance.

The recent development of a sudden intense source of ultraviolet light by Rentschler has made it possible to test this type of stimulus. The brief but intense dosage, which for convenience can be called a flash, is sufficient to kill small organisms.

Rentschler, Nagy, and Mouromself (1941) have found that the reciprocity law holds for ultraviolet killing of bacteria when the time is varied from a few microseconds (the duration of a flash) to several hours and Rentschler and Giese (1941) believe the same to hold for *Paramecium*. These animals are killed instantly by a flash with almost immediate bleb formation (vesiculation) and cytolysis (Fig. 1, A and B). Moreover, reducing the intensity of a flash reduces the injurious effect of the ultraviolet and produces a sequence of changes quite comparable to those observed with longer exposures to a source of constant low intensity.

The present investigation was undertaken to determine whether such a sudden intense dose of ultraviolet will cause contraction in muscle, conduction in nerve, and affect protoplasmic rotation, ameboid movement, oscillatory and ciliary movement, myonemes, and bioluminescence. Lethal changes in a number of small organisms have also been observed.

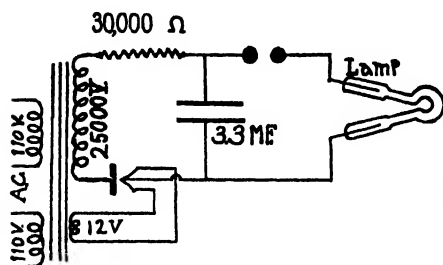
### Method

The outfit<sup>2</sup> for high intensity ultraviolet dosage is a quartz sterilamp through which is suddenly discharged a condenser at high voltage by the breakdown of an air spark gap in series with the lamp, as shown in Text-fig. 1. The condensers had a capacity

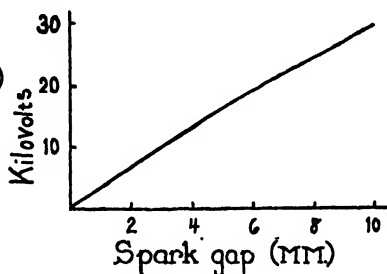
<sup>2</sup> I am deeply indebted to Dr. H. C. Rentschler, Director of the Research Laboratory, Westinghouse Lamp Works, Bloomfield, New Jersey, for the loan of his apparatus.

(C) of 3.3 microfarads and the spark gap was usually set at 6.1 mm., equivalent to 21,000 volts. The electrical energy,  $0.5 CV^2$ , is 728 joules. Lower voltages with less intense flashes are obtained by setting the spark gap closer. The relation is given in Text-fig. 2.

Spectra<sup>3</sup> of the discharge are reproduced in Fig. 1 and can be compared with the same lamp run on 60 cycle, 3,000 volt A.C., where the 2537 line is the effective ultra-violet radiation. It will be observed that the flash is very intense and almost continuous in the visible and near ultraviolet, that it presents many lines in the far ultraviolet but completely lacks 2537. The plates were not very sensitive to radiation below 2200 although there is considerable of this in the flash. In addition a number of lines are reversed, particularly at 2880 and in the region 2520. As the voltage of the discharge is reduced, there is less of a continuous spectrum but many widely separated lines appear, including those of the sterilamp. The figure also shows the



TEXT-FIG. 1



TEXT-FIG. 2

TEXT-FIG. 1. Diagram of connections for high intensity flash of ultraviolet light.

TEXT-FIG. 2. Relation between voltage and spark gap.

absorption of a 0.17 mm. glass cover slip, a 0.99 mm. glass microscope slide, and a 0.09 mm. cellophane sheet, of the type wet by water and somewhat transparent to lethal ultraviolet. The commercial treatment of cellophane to make it waterproof increases greatly ultraviolet absorption.

The quartz tube (1 cm. in diameter) of the sterilamp is bent in a ring (3 cm. outside diameter) around the microscope objective and adjusted about 5 mm. from the material on the slide, so that the cells or organisms are quite near the intense source of ultraviolet light of large area. Frequently a copper mosquito gauze (0.25 mm. diam. wire) with squares 1.7 mm. across is placed between the lamp and material. This is connected with the microscope and grounded to prevent an electrical field from disturbing the cells and organisms. Such a gauze casts no shadow since it is immediately under a large area of light. It does reduce the intensity somewhat in the ratio of the projected wire area to the total area.

<sup>3</sup> Dr. A. M. Chase of Princeton University collaborated in taking the ultraviolet spectra. A Jobin and Yvon quartz spectrograph kindly loaned by Alfred L. Loomis, Tuxedo Park, New York was used.



That the screen is effective can be seen from the fact that muscle and nerves are stimulated by the pulses of current in the lamp while the condensers are charging unless the grounded screen is present. The screen will also prevent the tearing off of a silver sputtered surface, which regularly happens when the mirror is near the lamp even though it is protected from ultraviolet rays by glass. The effect is probably due to eddy currents, which heat the silver to the point of volatilization.

The procedure is usually as follows: The cells are mounted in depression slides and covered with quartz cover glasses (0.2 to 0.5 mm. thick) taking care to exclude air bubbles. It is important to cover the drop of fluid, as the building up of a high potential on the lamp induces an opposite charge on the water of the slide, thereby lowering the surface tension at the air-water interface according to the well known principle that the greater the charge on a curved surface, the less the interfacial tension. When the discharge occurs, the sudden increase in surface tension results in violent stirring movements of the fluid. These were found to last about 0.06 second by taking photographs of animals in the uncovered drop at a rate of 100 per second.

An experiment is first made, covering the slide with 1.1 mm. of glass and with electrical shielding. This will disclose any effect of the visible and near ultraviolet. A second experiment with the shield removed will disclose any electrical effects and a third with the glass removed, the ultraviolet effects. It is impossible to observe what happens during a flash since the blinding light and sound of the spark cause involuntary winking, but the observations can be made as soon as the closed eyes are opened.

### *Muscle and Nerve*

No visible contraction of frog skeletal muscle or stimulation of sciatic nerve could be obtained with the highest ultraviolet flash intensity (25,000 volts) even though the electrically shielded thigh muscles and exposed nerves were very near the lamp or actually hung through the loop of the lamp. It is possible that ultraviolet was absorbed by the sheath of the nerve or fascia of muscle or that only a few surface fibers were stimulated, whose contraction could not visibly move the mass of inert muscle tissue. That this is the case is indicated by recent experiments (Harvey and Sichel, 1941) in which single muscle fibers or small bundles of muscle fibers were exposed to flashes of ultraviolet light and their movement automatically recorded on moving film. The fibers do not respond with a twitch but in 50 per cent of the flashes show a contracture. These flashes of ultraviolet light, like low intensity ultraviolet (Spealman and Blum, 1933), appear to act directly on the contractile substance rather than on the excitatory mechanism. The response is quite different from that to electrical stimuli.

### *Protoplasmic Rotation (Cyclosis)*

The earlier workers (*cf.* Ewart, 1903) on cyclosis noted that protoplasmic rotation would stop if the cells were stimulated mechanically, electrically, or in other ways and that a wave of shock stoppage would pass over the cell.

They found that electrical stoppage showed many phenomena similar to stimulation of muscle and nerve, obeying such classical laws as those of DuBois-Réymond and Pflüger, and giving electrotonus effects. The stopping of cyclosis may be considered analogous to stimulation in other cells.

*Nitella fragilis* is particularly good material to work with, since an action potential appears on stimulation shortly preceding the stoppage of cyclosis (Hörmann, 1898; Auger, 1931; Frank and Auger, 1932). However, I have noted that a *Nitella* cell showing no cyclosis would give an action potential on stimulation. Hill (1941) has recently studied the relation between action potential and cyclosis and finds that cyclosis always stops if an action potential appears but that an electrical stimulus too weak to elicit even a local action potential can stop streaming.

The *Nitella* cells used in this study were obtained from a brook near Kingston, N. J., and kept in the laboratory several days to a few weeks at a temperature of 28°C. Only young cells largely free of encrusted diatoms were used. They all showed rapid cyclosis but their size (0.25–0.4 mm. diameter, 3–30 mm. long) is such that ultraviolet light cannot be expected to penetrate throughout the cell. Indeed a flash of ultraviolet was frequently observed to stop cyclosis near the upper surface of the cell where the ultraviolet light entered, but not below. The cyclosis may also continue in a part of the cell under glass, when it has been stopped in that part under quartz. It is always necessary to shield the cells from the electric field with grounded copper gauze since without the gauze shock stoppage of cyclosis often occurs due to electrical stimulation.

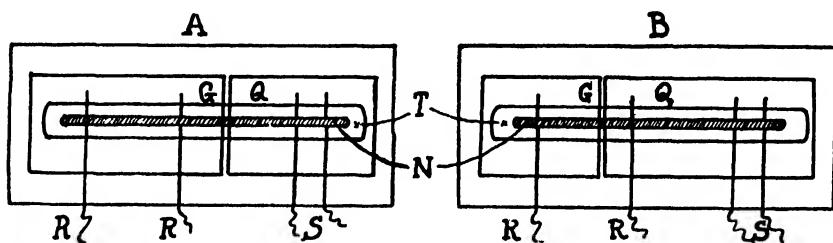
When the material was electrically shielded and covered with 1.1 mm. glass there was no effect whatever of an intense flash (22,000 volts) but when 0.5 mm. of quartz covered the cell, rotation stopped immediately. Sometimes there was no return of cyclosis and the cell died with its protoplast shrunken from the cell wall; frequently there was a return of slow or rapid rotation. The cell might live or might finally die. Occasionally a cell was not affected at all by an intense flash of ultraviolet and showed rapid rotation next day, just as did the controls. These were old cells encrusted with diatoms, which absorb the ultraviolet.

Permanent stoppage of cyclosis is not evidence of stimulation but of injury and death. By using ultraviolet light from a discharge with shorter spark gap, one can regularly obtain reversible stoppage of cyclosis. Thus with a 3 mm. gap (11,000 volts) there was no effect on cyclosis; with a 4.15 mm. gap (14,700 volts) the cyclosis became slowed and then recovered while with a 5.17 mm. gap (18,000 volts) the streaming stopped completely and then moved again within 2 minutes.

The fact that a flash of ultraviolet will stop cyclosis in an electrically shielded cell with later recovery, is definite evidence of stimulation by ultraviolet.

The important question is whether an action potential appears and whether it is propagated. The arrangement for determining these points is illustrated in Text-fig. 3.

A shows the method of detection of a propagated potential. A long cell of *Nitella* (0.4 mm. diameter  $\times$  32 mm. long) is laid over two nichrome wires (*R, R*) embedded in a paraffin trough (*T*) and connected to a Rubicon galvanometer.<sup>4</sup> Sometimes the end of the cell over one electrode is bathed in 0.01 M KCl solution, kept in place by vaseline. A 1.1 mm. glass sheet is placed between this half and the ultraviolet lamp. The other half of the cell is exposed to the ultraviolet since it is covered with 0.5 mm. quartz. This half is in contact with two nichrome stimulating electrodes (*S*) to test the irritability of the cell with make and break induced shocks. If an excitation



TEXT-FIG. 3. Arrangement of *Nitella* for detecting (A) propagated excitation and (B) local excitation. *S*, stimulating electrodes for testing irritability of cell. *R, R*, recording electrodes leading to galvanometer to detect action potentials. *G*, glass and *Q*, quartz cover slips. *T*, trough in paraffin to hold *Nitella* cell, *N*.

which is propagated appears from exposure to ultraviolet it should be detected as an action potential by a swing of the galvanometer.

In *B*, one of the detecting electrodes is also under quartz so that a non-propagated potential can be detected also. Unfortunately exposure of one nichrome wire (in water without the *Nitella* cell) to an ultraviolet flash (through quartz but not through glass) makes it negative electrically to another not exposed, giving a slight deflection of the galvanometer in the same direction as an action potential but so much smaller that there is little trouble in distinguishing the two.

Because of the sensitivity of the galvanometer, the *Nitella* cell was enclosed in a tin cigarette box with wire gauze soldered over the region exposed to the lamp and leads to the galvanometer shielded. These leads as well as the box and microscope were all grounded. Only the slightest movement of the galvanometer from pick up occurred during a discharge and there was

<sup>4</sup> Sensitivity 0.00077  $\mu$  a/mm.; resistance 1981 ohms; period, 4.1 sec.; critical external damping resistance 4800 ohms.

no escape of current from the stimulating electrodes that affected the galvanometer.

When the *Nitella* cell set up in trough *A* had recovered from handling so that cyclosis was rapid, a make and break induced shock was given. This stopped cyclosis and set up an action potential easily detected with the galvanometer. Cyclosis began again in 2 to 3 minutes and in 5 minutes was rapid. Then a 20,000 volt flash was given through 1.1 mm. glass. There was no effect on cyclosis and no action potential appeared. The glass was then removed and a second 20,000 volt flash given. The ultraviolet stopped the cyclosis under the quartz, but ordinarily there was no propagated action potential. However, in five instances out of fifteen trials propagated action potentials appeared which could not be attributed to other causes than ultraviolet stimulation.

If a *Nitella* cell, set up as in Text-fig. 3 *A*, was continuously illuminated with ultraviolet light from the lamp run on 3,000 volt, 60 cycle A.C., cyclosis in that half of the cell covered by quartz gradually slowed and after 4 minutes had practically stopped while the cyclosis in the half under glass was still apparent. In some cases no action potentials which were propagated appeared during or after the exposure. On the other hand, certain cells were particularly sensitive and rhythmic action potentials appeared during and after exposure to ultraviolet light, as often as once every 1.5 seconds. The cyclosis stopped abruptly on appearance of a potential.

When the arrangement was as in Text-fig. 3 *B*, again in some experiments no rhythmic action potentials appeared. The cyclosis stopped under quartz but was proceeding under the glass. We might expect that the ultraviolet radiated region would become negative to a non-radiated region but no such effect was observed. In other cases ultraviolet irradiation gave rise to definite rhythmic action potentials which immediately stopped cyclosis. These may be compared to the rhythmic potentials obtained from *Nitella* after chloroform treatment (Osterhout and Hill, 1929-30).

In eight flash trials with the *B* arrangement of electrodes, only three certain action potentials appeared although the cyclosis was always reversibly stopped or slowed by the ultraviolet flash. All cells were in good condition, since an action potential and stopping of cyclosis regularly occurred on electrical stimulation. Cells used for either the *A* or *B* experiments were alive next day and had suffered no irreversible changes; only those receiving long treatment with low voltage ultraviolet were dead.

We therefore conclude that an action potential may or may not appear at a region of *Nitella* exposed to a sudden flash of ultraviolet light and that it may or may not be propagated as an excitation wave, but the cyclosis is always either stopped or markedly slowed. Depending on its intensity, a flash of ultraviolet light behaves just as an electrical stimulus which may

also in *Nitella* slow or stop cyclosis with no action potential if the stimulus is weak, or stop cyclosis with a local or a propagated action potential if the stimulus is strong.

*Elodea canadensis* is also good material for cyclosis studies. Heilbrunn and Dougherty (1935) noted that small doses of ultraviolet increase and large doses decrease the rate of protoplasmic rotation in this plant. The leaves used in these experiments were made up of two layers of cells, large (100–150  $\mu$  long  $\times$  30  $\mu$  wide  $\times$  30  $\mu$  thick) on the upper surface and small (100–150  $\mu$  long  $\times$  15  $\mu$  wide  $\times$  15  $\mu$  thick) on the lower surface except near the midrib, where several layers were present. Looked at in cross-section of the living leaf the cylindrical cells are quite turgid. If the small layer of cells is toward the lamp, the ultraviolet light would have to pass 15  $\mu$  of cellulose wall, protoplast containing nucleus and chloroplasts, large sap vacuole, another layer of protoplast and cellulose to reach the large layer of cells underneath. Under proper conditions protoplasmic rotation is marked in both layers of cells and is stopped by a flash of ultraviolet light. We therefore have in this plant a means of estimating absorption of ultraviolet.

It was observed that when the large layer with active protoplasmic rotation was next to the lamp an intense flash of ultraviolet would permanently stop protoplasmic rotation in the large layer but the small cells underneath showed cyclosis, even after 3 hours. If the large cells were underneath so that the ultraviolet had to pass the small layer, there was no stopping of rotation in the large cells. Therefore only 15  $\mu$  of *Elodea* material is sufficient to absorb and prevent lethal effects under these conditions.

On decreasing the ultraviolet intensity by making the spark gap less, no intensity was found that would reversibly stop cyclosis. A 4.15 mm. gap (14,700 volts) had no effect and a 4.83 mm. gap (17,000 volts) slowed the rotation within 2 minutes and it stopped permanently in 3 minutes. With continuous exposure to the lamp run on 3,000 volts a.c. the rotation gradually slowed and stopped within 2 minutes. There was no recovery. Action potentials of these cells were not studied.

#### *Ameboid Movement*

Heilbrunn and Dougherty (1933) have studied the effect of ultraviolet light on *Amoeba proteus* and *A. dubia*, finding that the former, which is practically all plasmagel, liquefies, whereas the latter, practically all plasmasol, decreases in viscosity and then gels.

*Amoeba proteus* was used in these experiments. An intense (21,000 volts) flash on an *Amoeba* shielded from the electrostatic field and protected by 1.1 mm. of glass from ultraviolet light caused only a stopping of movement with immediate protrusion of a pseudopod in another direction, the effect of visible light as described by Mast (1931); through quartz alone the *Amoebae*

rounded up in whole or in part, the pseudopods largely disappeared, and adhesion to the slide was interfered with. The animals gave jerky movements and often became quite spherical (see Fig. 1, C and D). They were obviously liquid, and granules within them could be seen in moderate Brownian movement. Such animals never recovered, although their surface boundary was still distinct after 18 hours. In some *Amoebae* the surface boundary broke, with flow of the contents into the water.

With shorter spark gaps, an intensity of flash could be found (4,500 volts) which had no effect on *Amoeba* protected by glass but which stopped amoeboid movement instantly without glass. Recovery was rapid and the *Amoebae* were soon progressing again. With an 8,200 volt flash the liquefaction was very marked and long lasting.

Exposed to the same lamp run on 3,000 volts 60 cycle A.C. similar changes occurred over a period of a minute. The reversible effects in *Amoeba* are characteristic of mechanical and electrical stimulation and ultraviolet light can certainly be said to stimulate this animal.

#### *Oscillatory Movement*

The blue green alga, *Oscillatoria*, and the sulfur bacterium, *Beggiatoa alba*, both long thin filaments often coiled and twisted in a mass, undergo slow wavy motions or sinuous progressive movements resembling a freight train. The cause of the movement is unknown, although in *Beggiatoa* it has been attributed to flow of protoplasm outside of the cell as in a diatom, a caterpillar tractor effect.

An intense flash of ultraviolet did not stop the wavy motions of long filaments which projected from the coiled mass although they were completely exposed to the ultraviolet flash except for the buried end, which might have been protected. Completely isolated and exposed filaments showed less marked movement before exposure to the ultraviolet but these moderate movements were stopped permanently by a flash. The evidence seems to indicate that a filament can move after ultraviolet treatment if only one end of it is protected but not if it is wholly exposed.

#### *Ciliary Movement*

Ciliary movement is often independent of stimulation. However, in free swimming organisms some cilia appear to be under the control of the organism and stop beating on nerve stimulation. In a few instances cilia on epithelia can be started by mechanical stimulation if they have been slowed or stopped by adverse conditions such as acid or cyanide. The cilia of the frog's pharynx are likewise started by irritating substances which set up a reflex through sympathetic nerves, while parasympathetic fibers slow the ciliary motion.

Nervous control of the cilia of the mollusc gill is not yet certain (Lucas,

1931). Usually these cilia are beating rapidly and are not stopped by any kind of stimulation. However, in the clam (*Venus*) they are immediately rendered immobile by a flash of ultraviolet light (21,000 volts) all stopping at the end of the forceful stroke, irrespective of the position of the metachronal wave, as shown in Fig. 1 F. There was no stopping when protected by glass. The effect was not due to lack of oxygen, for cilia on under layers of the gill to which the ultraviolet cannot penetrate were still moving and on removal of the quartz cover slip the immobilized cilia did not start beating again, even after an hour.

Lesser doses of ultraviolet also stopped the cilia but more time was necessary. For example, with 12,000 volts the flash had no immediate effect but in 3 minutes the ciliary movement was somewhat slowed; with 14,000 volts the cilia quickly slowed and were stopped in 3 seconds. There was no recovery in 15 minutes.

When exposed to ultraviolet from the lamp run on 3,000 volts A.C. the rapidly beating cilia gradually became slower and slower and had practically stopped in 3 minutes, always at the end of the forceful beat. There was no recovery. These effects of ultraviolet light are obviously lethal and not to be considered a response to stimulation.

Cilia and flagella are so small that they may be expected to readily absorb ultraviolet and one universal observation is the cessation of such movement in cells, whether the organism itself goes to pieces as the result of the ultraviolet flash or not. Thus *Euglena*, *Volvox*, *Chilomonas paramecium*, *Spirillum volutans* and *Arbacia plutei* stopped moving instantly after an ultraviolet flash while the cells remained undisintegrated. With dark field, some flagella were observed attached to *Euglena* but in most they were not visible, although they are also difficult to see in the living *Euglena*. *Spirillum* was more resistant to ultraviolet and organisms could be seen moving slowly after a flash. Their flagella could be observed with dark field, waving so slowly as not to propel the organisms. *Chilomonas paramecium* stopped instantly after an ultraviolet flash and went to pieces within a few minutes with bleb formation. None of these organisms were affected by the light which will pass 1.1 mm. of glass.

The ciliates are universally destroyed by a strong ultraviolet flash (21,000 volts) the exact consequences depending on the intensity of the ultraviolet and size of the animal. Thus *Stylonychia*, *Chilodonella*, *Coleps*, *Urocentrum turbo*, *Paramecium bursaria*, *Epistylis plicatilis*, *Stentor coeruleus*, *Bursaria truncatella*, and *Frontonia* went to pieces within a few seconds after a flash.

The *Bursariae* observed were 350  $\mu$  wide and 480  $\mu$  long and the ultraviolet affected only one side of the animal which disintegrated, leaving moving cilia on the other side and in the oral groove. The coiled nucleus was half exposed and the animal rotated in circles, later becoming practically spherical

but living for some time. None of the above organisms were affected by the light from an intense flash (21,000 volts) which will pass glass.

Multicellular animals are not so easily affected. Rotifers may be observed moving about actively when Infusoria are disintegrated. The rhabdocoele, *Stenostomum*, which moves by cilia and contracts by muscles stopped instantly after the flash and in a few seconds had disintegrated on one side (where the ultraviolet had struck) and was soon a mass of debris (Fig. 1 E). *Cyclops* and nauplius larvae moved for a few minutes but were soon affected, *Daphnia* was more resistant but eventually died, while chironomid larvae and mites were very active for a long time. We may expect large forms covered with chitin to be quite resistant to an intense dose of ultraviolet light.

*Hydra fusca* and *Plumatella* sp., a fresh water Bryozoan, were quite unaffected when protected from a flash (21,000 volts) by electrical shielding and glass. When the glass was removed a flash caused immediately retraction in both animals with subsequent expansion. The hydras were active and the plumatellas looked normal for several hours but within 16 hours both were dead although controls not exposed to ultraviolet were alive and active. In these forms there is either reflex contraction or a direct effect of the ultraviolet on muscles of the animals.

A single flash of ultraviolet light caused contraction and breaking up of the chlorophyll bands of *Spirogyra*, prevention or slowing of cleavage of fertilized *Arbacia* eggs, and the formation of fertilization membranes, which pushed out on one side only, in unfertilized *Arbacia* eggs.<sup>5</sup>

### *Myonemes*

An 18,000 volt flash did not affect *Vorticellae* in any way if protected by glass but without glass the animals immediately contracted and expanded several times after the flash. The cytosome was retracted also. With lower intensities the contraction was delayed somewhat and the animals kept contracting and expanding as if irritated. A similar behavior was noted on continuous exposure to 3,000 volts A.C. excitation of the lamp. After some seconds a series of contractions occurred whose relaxation became increasingly difficult. The animals finally stopped moving and the stalk slowly went into the contracted condition.

*Epistylis* behaved in a similar manner, retracting the cytostome and becoming a round ball which only partially expanded after the more intense flashes. The peristomal cilia could be seen beating within. *Stentor coerulesus* also rounded up after a flash.

There can be no doubt of the response of myonemes to ultraviolet light.

<sup>5</sup> Observations in collaboration with Dr. Ethel B. Harvey.



*Bioluminescence*

There are two processes in luminous bacteria which can be observed, the luminescence and the movement due to flagella. Two forms<sup>6</sup> of bacteria in 3 per cent NaCl with phosphate buffer, mounted on depression slides under quartz<sup>7</sup> and under glass were observed. One, *Achromobacter fischeri* is not as brightly luminescent as *A. harveyi*; both are motile. The results were:

	Before flash (21,000 volts)	After flash
<i>A. fischeri</i> under glass.....	Fair light, motile	Fair light, motile
" " " quartz.....	" " "	No light, non-motile
<i>A. harveyi</i> " glass.....	Good " "	Good light, motile
" " " quartz.....	" " "	Very faint light, non-motile

Thus a single flash of intense ultraviolet is able immediately to reduce the luminescence intensity, as does the prolonged exposure to weak ultraviolet so carefully studied by Beijerinck (1916), Gerretsen (1915, 1920), and Giese (1941).

## SUMMARY

A study has been made of the effect of sudden intense flashes of ultraviolet light, acting on a wide variety of cells and tissues, with special reference to stimulation.

The flashes are obtained by a high voltage condenser discharge through a quartz mercury vapor sterilamp, using the method of Rentschler. The lethal effect of a single such discharge is widespread among unicellular organisms.

Medullated nerves and whole muscles are not visibly stimulated, because of absorption by connective tissue. Single muscle fibers undergo immediate contracture in 50 per cent of the experiments.

*Nitella* cells are stimulated, the effect depending on the dosage. Weak ultraviolet flashes slow or stop cyclosis reversibly. Strong flashes stop cyclosis reversibly with the appearance of a local or a propagated action potential. Very strong flashes kill the *Nitella* cells.

The effect of single flashes on myonemes, oscillatory movement, ameboid movement, cilia, flagella, and bioluminescence is described in the text.

<sup>6</sup> I am deeply indebted to Dr. F. H. Johnson for the species of bacteria and for aid in the observations.

<sup>7</sup> The fused quartz of the lamp is brightly phosphorescent after a flash as are the fused quartz cover glasses. These had to be removed to detect luminescence or darkness in the bacteria. Crystal quartz is not phosphorescent.

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## EXPLANATION OF PLATE 5

FIG. 1. Spectrum of ultraviolet flash. Slit width 0.1 mm. Scale at top gives wave lengths. Above and below the eleven numbered spectra is a mercury line spectrum from a quartz sterilamp run on 3000 volt 60 cycle A.C. (40 seconds exposure).

- (1) One flash discharge, 21,000 volts.
  - (2) One flash discharge, 21,000 volts through 0.17 mm. glass cover slip.
  - (3) One flash discharge, 21,000 volts, through 0.99 mm. glass slide.
  - (4) Two flash discharges, 15,500 volts.
  - (5) Four flash discharges, 11,000 volts.
  - (6) Twenty flash discharges, 4500 volts.
  - (7) Two hundred flash discharges, 1500 volts.
  - (8) Four flash discharges, 21,000 volts.
  - (9) One flash discharge, 21,000 volts through 0.09 mm. cellophane.
  - (10) One flash discharge, 21,000 volts of a krypton filled lamp.
  - (11) Krypton lamp run on 3000 volts, 60 cycle, A.C., 400 seconds exposure.
- (A) Rapidly moving normal *Paramecia* photographed with a 9000 volt flash, protected with glass.
- (B) *Paramecia* photographed immediately after a single 21,000 volt flash of ultraviolet light.
- (C) *Amoeba proteus* before and (D) immediately after a 21,000 volt flash.
- (E) The rhabdocoele, *Stenostomum*, immediately after a 21,000 volt flash. Note disintegration on one side (toward the ultraviolet light).
- (F) Edge of the gill of a clam (*Venus*) immediately after a 21,000 volt flash, showing immobilization of the cilia at the end of the forceful stroke.

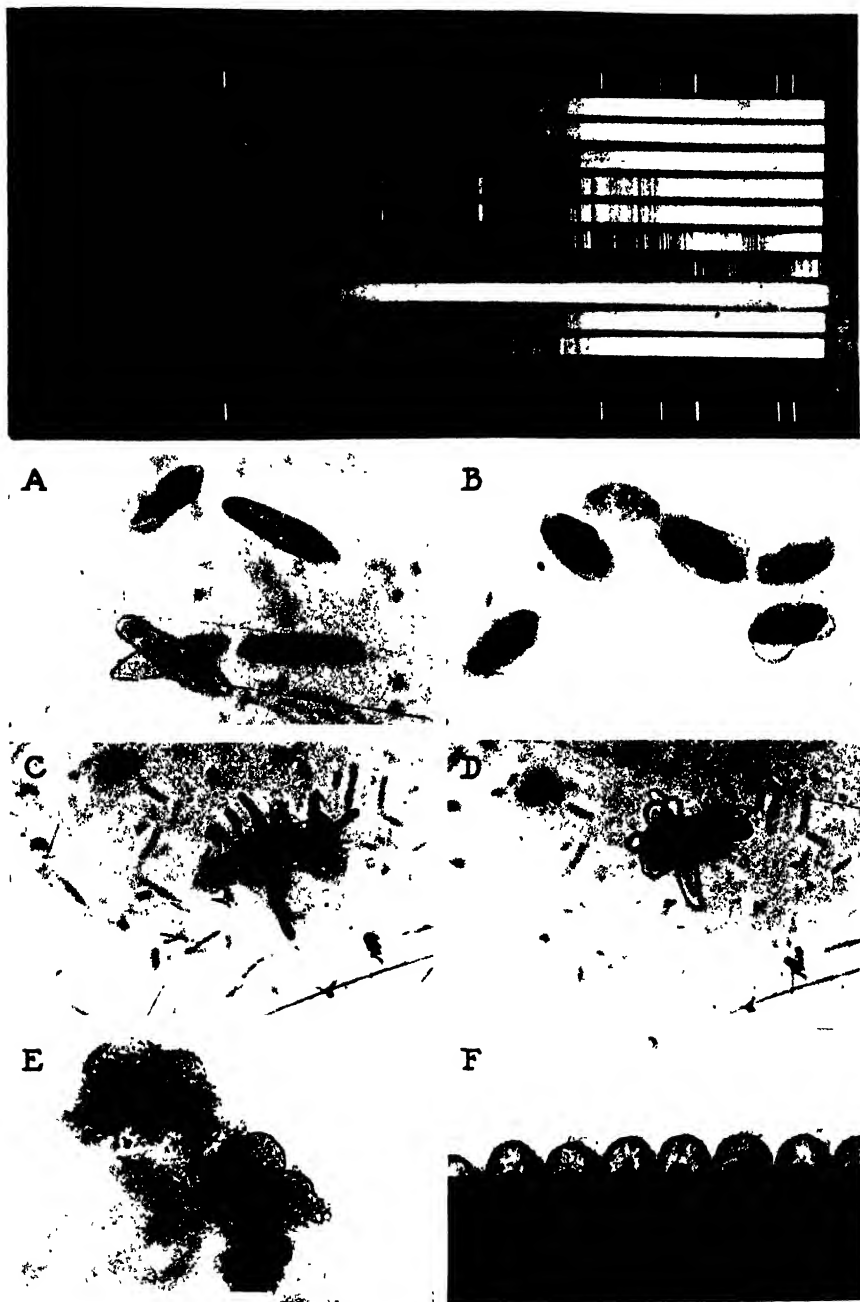


FIG. 1

(Harvey: Cell stimulation by intense flashes of ultraviolet light)



## A SIMPLE TIME WEIGHT RELATION OBSERVED IN WELL NOURISHED RATS

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The newer knowledge of nutrition as applied to the general physiology of growth suggests a fundamental re-evaluation of previous growth data and previous growth formulations. A careful analysis of the situation (Zucker *et al.*, 1942) with particular reference to the rat, the species most intensively studied by nutrition workers, has led us to two general conclusions:

1. The only experimental conditions which can possibly lead to growth according to any relatively simple law require a diet which is adequate for the most rapid growth attainable by nutritional means. Under these conditions only can the growth curve be a pure expression of the inherent growth characteristics of the organism. If on the other hand the rate of supply of one or more of the long list of chemical compounds required for maintenance as well as growth is reduced below such a level, the resulting growth simply expresses an arbitrary limitation imposed upon the organism by the investigator (whether he realizes it or not). The rate of supply of each one of the essential food factors is indeed a separate variable of the growth problem, varying not only with the diet but also with time, because the requirements for various factors change as growth proceeds. It is only if the rate of supply fully meets or exceeds the maximum requirements at any age that these complicated nutritional variables will no longer affect the growth rate, and a tremendous mathematical simplification is then to be expected.

2. Most growth data in the literature which have been used by students of growth probably fail to meet good nutritional standards. The whole growth problem has been confused and more or less run into a blank wall by the repeated working over of the same old data. There is a fundamental flaw in those data. New data are needed which do meet reasonable nutritional standards, and a useful, if perhaps limited, start can be made with a single species—the rat.

Elsewhere we have made a detailed presentation of data on the growth of normal rats of our albino colony (Zucker *et al.*, 1941, and 1942); these data are shown graphically in Fig. 1, together with a mathematical formulation which has been found satisfactory. We have presented detailed evidence inde-

pendent of the exigencies of curve fitting with any particular equation for the separate treatment of pre- and post-weaning growth data on both nutritional and developmental grounds. Of the two equations which were then found to

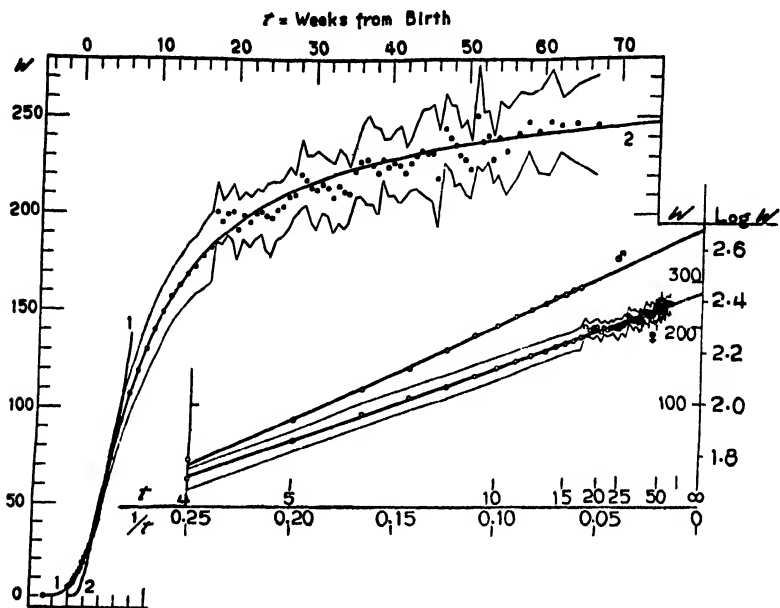


FIG. 1. The upper left curve is a weight time plot of the growth of female albinos of this colony; the axes are those indicated at the left and top of the figure. The lower right pair of straight lines are a log weight, reciprocal time plot of the same female data from 4 weeks on, and of our male data; the axes are those indicated on the right and bottom of the figure. The finer lines on either side of the data points in each case represent plots of  $W + \sigma$  and  $W - \sigma$  on a weight, or log weight, axis, as the case may be. With a normal frequency distribution of weights  $\frac{2}{3}$  of the observations would be included within the band so defined. In the weight time plot the spread is smaller than the diameter of the data points for the small initial weights.

Curve 1 in the weight time plot has the equation  $\log W = 2.88 \log T - 3.182$ ,  $T$  being days from conception. Curve 2 in the weight time plot, and also the straight line for the females in the log reciprocal plot, has the equation  $\log W = -\frac{2.84}{t} + \log 270$ ,  $t$  being weeks from birth.

fit the two postulated growth phases, the log log equation for the earlier phase has been successfully applied in other species as well (see MacDowell *et al.*, 1927 and 1930), but the log reciprocal equation which fits the data from 3 weeks on is apparently new (see footnote 3).

*The Log Reciprocal Equation*,  $\ln \frac{A}{W} = \frac{k'}{t}$ .—There can be no doubt as to the

practical value of the equation. It fits almost the entire life period very well, and it is simple enough to allow a direct plot of the data on a  $\ln W - 1/t$  grid (as shown in Fig. 1) characterized by a spread of the individual weights around the mean which remains beautifully constant at all ages. It defines two parameters:  $A$ , the value approached by the weight as time increases without limit and hence an inherent size factor, and  $k'$ , a growth intensity factor.  $k'$  is of course the slope of the  $\ln W - 1/t$  plot, and  $\ln A$  is the intercept (at  $1/t = 0$ ). As shown in Fig. 1 the equation has a point of inflection, and this is fixed at  $W = 0.135 A$ . The equation is of entirely empirical origin, but this fact does not of course have any bearing upon its possible general validity; whether it is merely a concise summary of these particular data or whether it has a wider applicability and any general biological significance can only be determined by further study.

*Comparison with Other Growth Equations.*—First let us see how this equation compares with other growth equations which have been suggested. Fig. 2 compares the fits to our female growth data obtained with various growth equations. This comparison is made on the ability to fit either the post-weaning data alone, or both pre- and post-weaning data. The questions posed are these:

1. In the past it has been found that most of these equations were not able to fit the entire life period, and it was necessary to assume growth cycles to explain the discrepancies. Is it possible that some of these equations can fit the entire life period, thus making the assumed cycles unnecessary, if nutritional distortions are absent?

2. Assuming that our analysis into two phases of growth is valid, how do the various equations compare in ability to fit the post-weaning data representing the second phase? In this comparison it seems that we are applying the equations of Backman, Hatai, Gompertz, Brody, Pearl, and Bertalanffy in the manner in which they are usually applied to growth data. Crozier and Robertson would presumably apply their equations very differently, since they have offered cyclic analyses of growth which break up the post-weaning weights into portions separately fitted to their equations. It must therefore be understood that we are investigating the properties of their equations, and not of their complete formulations involving equations and cyclic analysis.

The equations are listed in Table I in various forms which may be of interest. It may be noted that almost every one of these equations is "theoretical," in the sense that it derives in some way from a theory of growth, or can be provided with some sort of rationalization; most of the constants in the equations have independent meanings in terms of the various theories of growth (see summary in Zucker *et al.*, 1942), but their numerical values are not predictable from the theory, and they are therefore treated as adjustable constants in fitting data. A few equations listed in Table I have not been applied in Fig.



2. The equations of von Hoesslin and Fischer have very unsuitably late points of inflection, and have not been tried for this reason. The Robertson equation has been fitted in spite of its late point of inflection because of its historical interest and its widespread acceptance even today as *the* theoretical growth

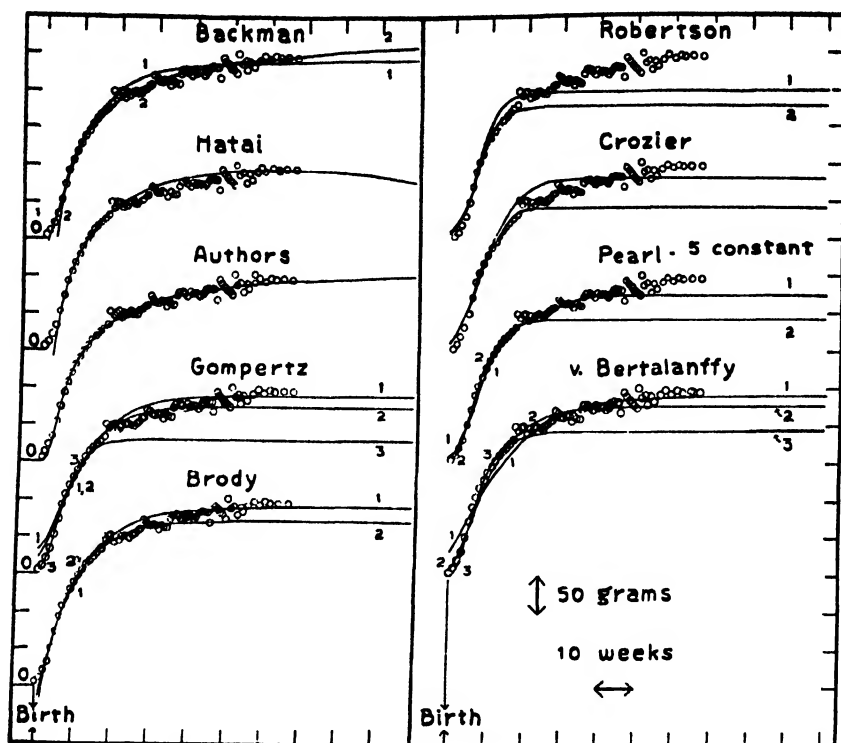


FIG. 2. Comparison of the ability of various growth equations to fit the growth of our female rats. The data are plotted as weight against time. References to the various equations are given in Table I. Where more than one curve is plotted and the curves cross or come together, they are numbered at critical points. In the case of the Backman equation, curve 2 is not the same form of equation as curve 1, but is a simplified form (see Table I). In all the other cases with more than one curve plotted, the different curves are compromise attempts to adjust the equation to various regions of the data.

equation. No attempt was made to fit Wetzel's equation, or Pearl's equation containing more than four terms in the power series, because of the large number of adjustable constants in both cases which make the equations very difficult to apply, and which make the interpretation of a good fit as contributory evidence of biological significance very doubtful. Snell (1929) has quite properly pointed out that Robertson's and Crozier's equations are incorrectly

derived from their common basic assumption that a chemical reaction determines the rate of growth, and has suggested corrected forms. We have not worked with these corrected forms principally because the correction is also in error, so that the "corrected" equations are in no way preferable to the originals. Snell's criticism is perfectly valid, but it is in the attempted application of the criticism that the error has crept in.

A few words of explanation seem to be in place since the point has apparently been passed over without notice. In the original derivations of Crozier and Robertson the expression for the rate of a chemical reaction was written in terms of weight of reactants rather than concentration, or weight/volume, as required by the laws of chemical kinetics. This is indeed a serious flaw in the rationale of both equations. Snell reasoned by analogy with the course of a chemical reaction going on in identical solutions in several different beakers that the desired rate of change of weight is given by the product of the volume and the known rate of change of weight/volume, or concentration. This analogy is not a proper one, because in the analogue the volume of the system does not change during the course of the reaction, as it does in the growing cells and body fluids. The proper expression for  $\frac{d(W/V)}{dT}$  is  $\frac{1}{V} \frac{dW}{dT} - \frac{W}{V^2} \frac{dV}{dT}$  which reduces to  $\frac{1}{V} \frac{dW}{dT}$ , the expression used by Snell, only when  $V$  does not change with time. Interestingly enough, if the proper expression for  $\frac{d(W/V)}{dT}$  is introduced, Snell's assumption that the rate of increase in  $V$  is proportional to the rate of increase in  $W$  leads to equations of the same form as the original uncorrected equations in both cases, the only difference being that the adjustable constants have different interpretations.

In judging the fit in Fig. 2, the range of variation of individual weights from the means shown in Fig. 1 should be borne in mind. On a weight time plot the spread is much smaller for small weights than for large weights. Considering this experimental variation, it must be concluded that no one of the equations in Fig. 2 provides an acceptable fit to both pre- and post-weaning data. Restricting consideration to the post-weaning data alone, one can see that only Backman's equation 2 and our equation provide fully satisfactory fits to the post-weaning data (see footnote 3).

*Fit of the Log Reciprocal Equation to Other Rat Data.*—Several other sets of rat growth data are available which approximate our nutritional requirements and which cover parts of the post-weaning period. Fig. 3 shows that the log reciprocal equation is competent to fit such data from other laboratories, on other colonies and diets. The fits are surprisingly good except for the nutritionally poor Donaldson data which were included for comparative purposes; even in this case the form of the deviations from the log reciprocal equation is in excellent agreement with our conception of the effect of the dietary inade-

TABLE I

Beckman	1		$\frac{dW}{dT} = e^{s_0 + s_1 \ln T + s_2 \ln^2 T}$	$\ln T_{\text{infl.}} = -\frac{s_1}{2s_2}$
	2	if $\left. \begin{matrix} s_2 = 0 \\ (s_1 + 1) < 0 \end{matrix} \right\} A \cdot W = \frac{e^{s_0}}{-(s_1 + 1) T^{-(s_1 + 1)}}$	$\frac{dW}{dT} = e^{s_0 + s_1 \ln T} = e^{s_0} T^{s_1}$	none
Metal		$W = a + bT + c \ln T$	$\frac{dW}{dT} = b + \frac{c}{T}$	$T_{\text{max.}} = -\frac{c}{b}$
Zucker		$\left(\frac{A}{W}\right)^2 = e^{k'} = 10^k$ or $\ln W = -\frac{k'}{T} + \ln A$	$\frac{dW}{dT} = \frac{kW}{T^2} = \frac{W}{T} \ln \frac{A}{W}$	$\left\{ \begin{matrix} W_{\text{infl.}} = 0.135 A \\ T_{\text{infl.}} = \frac{k'}{2} \end{matrix} \right.$
Gompertz		$W = A e^{-e^{-k(T-T^*)}}$ or $\ln \ln \frac{A}{W} = -k(T-T^*)$	$\frac{dW}{dT} = kW \ln \frac{A}{W}$ .	$W_{\text{infl.}} = 0.368 A$
Brody		$\ln(A-W) = -k(T-T^*) + \ln A$	$\frac{dW}{dT} = k(A-W)$	none
Robertson		$\ln \frac{W}{A-W} = kA(T-T^*)$	$\frac{dW}{dT} = kW(A-W)$	$\left\{ \begin{matrix} W_{\text{infl.}} = \frac{A}{2} \\ T_{\text{infl.}} = T^* \end{matrix} \right.$
Crozier		$\ln \frac{A(K_1 W + K_2)}{K_1(A-W)} = (K_1 + K_2 A)T$ or $\ln \frac{K_1/K_2 + W}{A-W} = (K_1 + K_2 A)T - \ln \frac{AK_2}{K_1}$	$\frac{dW}{dT} = K_1(A-W) + K_2 W(A-W)$ $= K_1 A + (K_2 A - K_1)W - K_2 W^2$	$W_{\text{infl.}} = \frac{1}{2} \left( A - \frac{K_1}{K_2} \right)$
Pearl		$W = \frac{A}{1 + e^{F(t)}}$ $F(t) = a_0 + a_1 t + a_2 t^2 + a_3 t^3 + \dots$	$\frac{dW}{dT} = -\frac{1}{A} W(A-W) F'(t)$	
v. Bertalanffy		$\ln(A^{1/2} - W^{1/2}) = -kt + \ln(A^{1/2} - W_0^{1/2})$	$\frac{dW}{dT} = 3kW^{3/2}(A^{1/2} - W^{1/2})$	$W_{\text{infl.}} = 0.297 A$
Fischer		$\ln \frac{A^{1/2}}{A^{1/2} - W^{1/2}} = k(T - T^*)$	$\frac{dW}{dT} = \frac{3k}{A^{1/2}} W(A^{1/2} - W^{1/2})$	$W_{\text{infl.}} = 0.422 A$
v. Moesslin		$W^{1/2} = A^{1/2} \left[ \frac{2T(r+t^*) - (r+t^*)^2}{T^2} \right]$	$\frac{dW}{dT} = 8A^{1/2} W^{3/2} [T - (r+t^*)]$ or $\frac{d(W/A)}{d(\frac{T}{T^*})} = 2(1 - \frac{T}{T^*})$	$\left\{ \begin{matrix} W_{\text{infl.}} = 0.512 A \\ W_{\text{max.}} = A \\ T_{\text{max.}} = T \end{matrix} \right.$
Wetzel		$\ln W = a + b e^{-\beta T} \cos(\theta T + c) + d e^{-\gamma T} \sinh(\omega T + t)$	$\lambda \frac{d^2 \varphi}{dT^2} + \rho \frac{d\varphi}{dT} + \frac{\varphi}{K} = \varepsilon + \varepsilon_0 e^{-\beta T} \cos(\theta T - \varphi)$ $\varphi = \ln \frac{W}{W_0}$	

## EXPLANATION OF TABLE 1

All variables are italicized.  $W$  is weight,  $t$  is age from birth,  $T$  is age from conception,  $A$  is the final weight,  $e$  is the natural base of logarithms. Natural logarithms are used throughout in order to avoid a conversion factor in the differential form. The last column shows the location of points of inflection and maxima in the weight time curve; conditions for singular points are too elaborate for tabular form in the Pearl and Wetzel equations, and the reader is referred to the original publications. *Backman*—see Backman, 1931, 1938 *a*, *b*; Zucker *et al.*, 1942; footnote 3 below. Backman has not published an integration for equation 1. The differential form can be fitted to data in the usual way for equations of the form  $y = a + bx + cx^2$  followed by graphic integration. Equation 2 is conveniently fitted by plotting  $\ln(A - W)$  for various assumed values of  $A$  against  $\ln T$  and determining the best straight line. *Hatai*—see Pearl, 1907; Hatai, 1911; Donaldson, 1924; Zucker *et al.*, 1942.

The equation is conveniently fitted by plotting  $\frac{dW}{dt}$  against  $1/t$  and determining the best straight line, followed by calculating the best average value of the integration constant. *Zucker*—see Zucker *et al.*, 1941, and 1942. Fitted by plotting  $\ln W$  against  $1/t$  and determining the best straight line. *Gompertz*—see Winsor, 1932; Backman, 1938 *a*; Lumer, 1937.

Fitted by plotting  $\ln \frac{A}{W}$  for various assumed values of  $A$  against  $T$  and determining the best straight line. Note that the Zucker equation predicts a straight line plot of  $\ln \frac{A}{W}$

against  $\ln t$ , rather than  $t$  or  $T$ . *Brody*—see Brody, 1925, 1926, 1927 *a*, *b*; Ludwig, 1929; Kaufman, 1930; Zucker *et al.* 1942. Fitted by plotting  $\ln(A - W)$  for various assumed values of  $A$  against  $T$  and determining the best straight line. Note that the Backman equation 2 predicts a straight line plot of  $\ln(A - W)$  against  $\ln T$  rather than  $T$ . *Robertson*—see Robertson, 1907-08, 1923, 1926; Pearl, 1924; Ludwig, 1929; Brody, 1927 *a*; Schmalhausen, 1929; Needham, 1931; Backman, 1931, 1938 *a*; von Hoesslin, 1930; Winsor, 1932. See Robertson, 1923, for methods of fitting. *Crozier*—see Crozier, 1926; Robb, 1929; Brody, 1927 *a*. The second form given for the integrated equation is very similar to the equation

$\ln \frac{B + W}{A - W} = KT + c$  often called the general logistic, used by Enriques, 1909, Robertson,

1926, and Lumer, 1937. The only difference is that Crozier's equation has only three independent adjustable constants as against four in the general logistic. Both equations have three independent adjustable constants in the differential form; in the Crozier equation the integration constant is evaluated in terms of the other three constants, making the condition that  $W = 0$  at zero time. *Pearl*—see Pearl, 1924; Brody, 1927 *a*; Ludwig, 1929. A method of fitting involving the use of selected points at equal intervals on the time scale has been worked out by Pearl, 1924. *von Bertalanffy*—see von Bertalanffy, 1934, 1938; Putter, 1920, Ludwig, 1929. Fitted by plotting  $\ln(A^{1/3} - W^{1/3})$  for various assumed values of  $A$  against  $t$ , and determining the best straight line. The equation differs from Brody's only in the exponent of  $A$  and  $W$ , but this difference introduces a point of inflection. *Fischer*—see Fischer, 1928; Ludwig, 1929. This equation is related to Robertson's in the same way that von Bertalanffy's is to Brody's. *von Hoesslin*—see von Hoesslin, 1926, 27, 1930, 1931, 1932, 1934; Zucker *et al.*, 1942. *Wetzel*—see Wetzel, 1932, 33, 1934, 1937; Zucker *et al.*, 1942. The constants in the differential form are those with meanings in terms of Wetzel's theory. The integrated equation written in terms of these constants is very long; it is written in Table I in simplest form, but with the same number of independent constants many of which are complex functions of the theoretical parameters which appear in the differential form, and of the two integration constants.

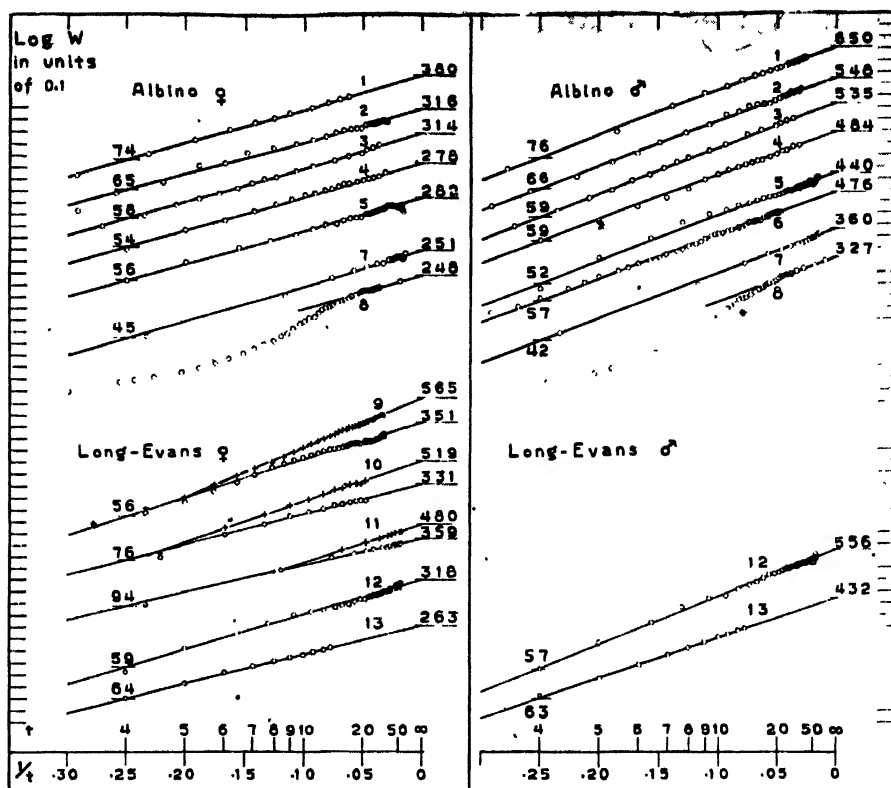


FIG. 3. Log reciprocal plot of the growth of various rat colonies on diets optimal for growth. 1, Mendel and Hubbell, 1935; 2, Smith and Bing, 1928-29; 3, Maynard, 1930; 4, Macy *et al.*, 1927, group labelled "stock diet"; 5, Freudenberger albinos, 1932; 6, Sperry and Stoyanoff, 1934, series III (detailed growth data kindly supplied by Dr. Sperry); 7, King, 1915; 8, Donaldson *et al.*, 1906; 9 and 10, Evans, 1923-24, series I and II (circles are controls and crosses are females repeatedly bred); 11, Hart and Cole, 1939 (circles are controls, crosses are females repeatedly bred); 12, Freudenberger Long-Evans strain, 1932; 13, unpublished data on the growth of our colony of Long-Evans rats in 1931 and 1932 on our regular stock diet.

All the data for one sex are plotted on the same reciprocal time scale, but in order to separate the lines the log weight scales are separated by 0.2 or 0.3 logarithmic unit. For orientation therefore we have written in on each line the values of  $W$  at 4 weeks and at  $\infty$  ( $\frac{1}{t} = 0, W = \Lambda$ ).

The original figures for mean weights were available for numbers 2, 6, 7, 8, 9, 10, 13. Plotted points in numbers 5, 11, and 12 are original data points read off the published graphs. Plotted points in numbers 1, 3 and 4 were read off the published smooth curves at suitable intervals, since the original data points were neither tabu-

quacies upon growth (Zucker *et al.*, 1941). The Donaldson rats are under the greatest nutritional growth inhibition during the early period when the inherent growth impulse calls for the most rapid growth and when nutritional requirements are therefore most critical. As the effect of the inherent growth impulse slows down, nutritional requirements become less critical, the same diet becomes gradually more adequate, and the rats gradually recover spon-

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lated nor plotted. The graphs which had to be depended upon as sources for many of these sets of data are unfortunately very small.

Freudenberger (numbers 5 and 12) did not reject animals which were losing weight from his means. King (number 7) did so, and we have done so with our data and with the data of Evans, who published his individual weights. We have no information on how the other data are constituted in this respect.

The King data are among the earliest. The diet—"selected table scraps"—is not very well characterized, but could be quite good by current standards. The points have a very unfortunate distribution on a log reciprocal plot because of the large intervals between weighings, and this makes it difficult to decide upon a fit.

We are presenting the evidence for the ability of the log reciprocal equation to fit these data in the form of a log weight, reciprocal time plot rather than a direct weight time plot because it appears from our data that this is the most generally satisfactory manner of presentation. It results in a straight line with the experimental error, or range of variation around the mean, covering a band of constant width throughout (see Fig. 1). Gray (1928) has emphasized a danger in the logarithmic plotting of weight data: "Where a process of growth involves the use of a logarithmic function it is very unwise to trust to graphical methods *unless it can be shown that the ratio of probable error to the mean is constant during the whole period of growth* . . . the only real test consists in a comparison of observed and calculated values." (Italics are ours.) Our data satisfy the qualifying requirement laid down by Gray, that the ratio of the probable error (or standard deviation) is constant during the whole period of growth. The constancy is only approximate, but any fluctuations are second order effects as far as the present discussion is concerned. Under these conditions a weight time plot, or a tabulation of calculated and observed weights as suggested by Gray, is an inferior method of comparison, because deviations are of constantly decreasing biological significance and must be mentally corrected at the hand of the information contained in a tabulation or plot of probable errors or standard deviations as a function of weight. Under our conditions it is precisely a logarithmic plotting of weight which provides the most balanced presentation of the data and their agreement with a particular curve; we have no reason to suppose that other data differ markedly from ours in this respect. Fig. 2 would be very much improved if it involved a log weight-time plot rather than the direct weight-time plot for just this reason; we chose the inferior presentation partly as a concession to custom, partly as a practical demonstration. It is necessary to emphasize this point because Gray has been widely quoted as showing that logarithmic plotting is without exception or qualification a biased unfair manner of presentation (Richards, 1935; Huxley, 1932; see also Davenport, 1926).

taneously. The late point of inflection and rapid cut-off found in several of the equations of Table I and Fig. 2 are quite characteristic of such nutritionally inhibited growth with spontaneous realimentation; in so far as these equations were designed to fit distorted growth curves they were made unsuitable for normal data.

The agreement in the case of the Long-Evans rats is of interest, for these rats are of a very different strain from our albinos. They are the descendants of a cross between one wild gray Norway male and a few laboratory albino females. King has reported in some detail on the growth of captive grays (King, 1939); these are descendants of wild Norway grays and therefore represent the other component of the Long-Evans strain. The data cannot be fitted by the log reciprocal equation, but it is possible that the diet is faulty; these animals were not raised on the diet previously used for King's albinos (King, 1915), which was composed of selected table scraps, but on a diet of cooked cereals. King specifically says that no attempt was made to provide a superior diet.

Of further interest is the presence among the available data obtained on known good diets of two examples of growth stimulation leading to "super-normal" growth which are also fitted by the log reciprocal equation. The Evans data are two series of females given daily injections of anterior pituitary substance. The Hart and Cole data represent the maximum stimulating effect of pregnancies upon growth; this was obtained by putting the rats through nine to ten successive pregnancies and lactations starting at 4 weeks of age, at which time precocious sexual maturity was induced by injecting gonadotropic hormone. This hormone has no effect on growth *per se*.

*The Parameters of the Equation.*—The values of the parameters for the several sets of data are presented in Table II. The albino colonies all show approximately the same  $k$  for each sex; this expresses itself in Fig. 3 in the fact that the lines are parallel. These colonies appear to differ only in mean size (A). The descendants of the cross between albinos and grays do not show a constant  $k$ , but the ratio  $\frac{k_{\text{male}}}{k_{\text{female}}}$  is in good agreement with that characteristic of the albinos. The obvious interpretation of these findings is that  $k$ , or rather the pair of values  $k_{\text{male}}$ ,  $k_{\text{female}}$  in a fixed ratio, are associated with the genetic constitution of the rat. At any rate the parameters defined by the equation not only can be given rational interpretations but are shown by the hand of data to provide an analysis of growth into two components of practical usefulness and significance.

The approximate constancy of  $k$  for female albinos, and indeed the absence of a break in the female growth curve as fitted by the log reciprocal equation seem to be in disagreement with the findings of Cole and Hart (Cole and Hart, 1938; Hart and Cole, 1939). These findings indicate that  $k$  is very greatly

increased by pregnancies over the value characteristic of unmated females; one would expect rats first bred at 17 weeks as ours are to show an immediate increase in  $k$ , producing a break, and one would also expect the mean  $k$  for different sets of data to be somewhat different, since colony practice with respect to the age of first breeding and frequency of breeding differs. Neither expectation is met; the female data behave in every way like the male data in which no disturbing effects are expected. It may be that breeding must be

TABLE II  
*The Parameters of the Log Reciprocal Equation*

No. (Fig. 3)		Females		Males		$\frac{k_{\text{male}}}{k_{\text{female}}}$
		k	A	k	A	
	Albinos					
	Authors' data	2.84	270	3.65	470	1.29
1	Mendel-Hubbell (1935)	2.84	380	3.73	650	1.31
2	Smith-Bing (1928)	2.76	316	3.67	548	1.33
3	Maynard (1930)	2.93	314	3.83	535	1.31
4	Macy <i>et al.</i> (1927)—group labelled "stock diet"	2.86	278	3.66	484	1.28
5	Freudenberger (1932)	2.80	282	3.70	440	1.32
6	Sperry-Stoyanoff, series III (1934)			3.68	476	
7	King (1915)	(2.98)	251	3.73	360	(1.25)
	Long-Evans					
9	Evans—normal I (1923)	3.19	351			
	Evans—pituitary I injected	4.21	565			
10	Evans—normal II (1923)	2.56	331			
	Evans—pituitary II injected	3.46	519			
11	Hart-Cole—normal (1939)	2.33	359			
	Hart-Cole—pregnant	3.32	480			
12	Freudenberger (1932)	2.93	318	3.96	556	1.35
13	Authors' data	2.46	263	3.34	432	1.36

$k$  was calculated for common logarithms, and is in units of weeks.  $A$  is in grams.

started very early, earlier than is customary in breeding colonies, and carried out intensively in order to affect  $k$  significantly.

*The Relation with Heterogony.*—The equation

$$\ln W_1 = K' \ln W_2 + \ln C'$$

is the well known law of relative growth (heterogony of Huxley, 1932).  $W_1$  and  $W_2$  are weights, or other measures of growth, of two organs or parts of the body observed at the same time (or of a part and the whole). The equation is equivalent to the statement that the specific growth rates  $\frac{1}{W} \frac{dW}{dt}$  of the parts



are all proportional to each other. It has been applied successfully to such a range of species and conditions that it ranks as a really well founded generalization in the field of growth. Whatever meaning one wishes to ascribe to it, whether it is exact or approximate, it closely represents the course of relative growth. It is therefore a distinct merit of the log reciprocal equation that it immediately suggests such a course of relative growth. This is also true of the log log equation used for pre-weaning growth and of the compound interest law sometimes used for growth. It is true of many other possible equations—of any equation of the form

$$\ln W = aF(t) + b$$

where  $F(t)$  can be the same for the growth of the different parts being compared; *i.e.*, where  $F(t)$  does not contain any of the growth parameters referring to the particular organ or part specifically. Since heterogony applies to such a wide range of species the possibility exists of many different time growth equations for different species or larger groups of species, differing in the form of  $F(t)$ . None of the other equations listed in Table I is of the required form. Any of these equations can of course be reconciled with heterogony by assuming that each organ or part has a different form of time law of growth<sup>1</sup> but other things being equal, a formulation which does not require all these different laws of growth for different parts is preferable. Thus the log reciprocal equation not only describes the course of body growth in time, but also the course of growth in time of the femur length (Hinkel, 1942), and of the femur weight, femur organic matrix, and femur ash (Zucker *et al.*, 1942; in these data good agreement is found up to 700 days of age in both males and females).

<sup>1</sup> Thus if  $W_1$  in the above equation is the body weight,  $W_2$  the organ weight, the form of growth of  $W_2$  in time for any of these equations is obtained by substituting for  $W_1$  its equivalent  $C'W_2K'$ . In the log reciprocal equation and in the others which suggest heterogony the resulting equation for  $W_2$  as a function of time is of the same form as the original equation for  $W_1$  as a function of time—for the others it is different. This procedure is a fundamental part of the derivation of Bertalanffy's and Fischer's equations; their theories lead to growth rate equations in body length rather than weight. To get an equation for weight as a function of time they replace length in the integrated equation by  $qW^{1/3}$ , limiting length by  $qA^{1/3}$ , assuming that  $K'$  in the heterogony equation has the value  $1/3$ . Lumer (1937) has concluded that heterogony is incompatible with sigmoid growth. The evidence given is that heterogony is not compatible with three particular sigmoid growth equations (Robertson, Crozier, and Gompertz), and the implicit assumption is made that the same equation must apply to the part and to the whole. The conclusions he draws are evidently far too broad, because there are sigmoid equations such as the log reciprocal which *are* compatible with heterogony assuming the same equation to apply in the growth of the whole and the part, and, for the other equations, it is not necessary to assume that the same form of equation applies to the part and the whole.

Recent students of heterogony have tended to dismiss the parameter  $\ln C'$  in the heterogony equation as an unimportant, uninterpretable constant (Huxley, 1932; Needham, 1934), and have centered attention upon  $K'$ , the proportionality constant relating the specific growth rates of the parts compared. Thus "the constant  $b$  [our  $C'$ ] is of little biological significance, since it merely denotes the value of  $y$  [our  $W_1$ ] when  $x$  [our  $W_2$ ] is unity" (Needham, 1934). Now if we write this integration constant  $\ln C'$  in its most general form, we have

$$\ln \frac{W_1^*}{W_1} = K' \ln \frac{W_2^*}{W_2}$$

where  $W_1^*$  and  $W_2^*$  are the values of  $W_1$  and  $W_2$  at some reference time. Needham chose the time at which  $W_2^* = 1$ —a choice which depends upon the units in which  $W$  is expressed, and which is therefore arbitrary as he pointed out. The law of relative growth derived from the log reciprocal equation has  $W_1^*$  and  $W_2^*$  interpreted as  $A_1$  and  $A_2$ , the limiting sizes of the parts compared. Now quite aside from the merits of the log reciprocal equation, this reference point would seem to recommend itself for relative growth. It has been frequently emphasized that there can be no significant reference point for heterogony at conception, or at any time during fetal life, for different parts and organs are first laid down at different times after conception; different parts of the body do not have a common beginning. (See MacDowell *et al.*, 1927; Schmalhausen, 1927, 1928; Huxley, 1932.) No intermediate point suggests itself. But the end, the final object of all the development and growth and change in shape and proportions surely has particular significance for relative growth as well as for time growth. The interpretation of the  $\ln C'$  parameter provided by the log reciprocal equation is thus reasonable and appealing in its own right. The heterogony equation can then be written

$$\ln \frac{A_1}{W_1} = \frac{k_1'}{k_2'} \ln \frac{A_2}{W_2}$$

*The Function  $\ln \frac{A}{W}$ .*—This function appears to be a useful measure of growth status, measuring the closeness of approach to the limiting or equilibrium weight independently of what that limit is. If two animals are chosen at random each weighing 200 gm., they are of course identical as far as weight is concerned, but their respective growth statuses may be very different, for the one animal may be an inherently smaller animal, much closer to its limit size, and hence nearer the end of the growth process than the other. This proposed growth status function makes the animals most nearly comparable when they have achieved the same percentage of their final limit weight. In terms of this

function, which we might call the growth function  $R$ , time growth and relative growth take the very simple forms

$$\begin{aligned} R_1 t &= k_1' \\ R_2 t &= k_2' \end{aligned} \quad R_1 = \frac{k_1'}{k_2'} R_2$$

or inverse proportionality between  $R$  and age, and direct proportionality between  $R$ 's for different parts of the body. The time derivative of  $R$  is  $-\frac{1}{W} \frac{dW}{dt}$ , the negative value of the specific growth rate.  $R$  is of course a quantity which decreases as the animal grows, approaching zero at the limit weight, so that its time derivative is of opposite sign from the time derivative of an increasing quantity like  $W$  or  $\ln W$ . The time rate equations then take the forms

$$\begin{aligned} -\frac{dR_1}{dt} &= \frac{R_1}{t} & \frac{1}{R_1} \frac{dR_1}{dt} &= \frac{1}{R_2} \frac{dR_2}{dt} \\ -\frac{dR_2}{dt} &= \frac{R_2}{t} \end{aligned}$$

Many growth equations introduce the limit weight  $A$  in the term  $(A - W)$ . This is by no means suitable as a measure of growth status, as can be seen from a consideration of the natural variation in  $W$  as  $W$  approaches  $A$ . The coefficient of variation of  $W$  is approximately constant throughout life (Zucker *et al.*, 1942); this means that a gram of weight gained or lost, or a gram difference between any two animals, is of less and less significance to the animals as they get larger, since it occurs with steadily increasing frequency, and in fact that the biological significance of weight differences is best measured on a percentage scale of  $W$ , or on an absolute scale of  $\ln W$ . This is reasonable on general grounds, when one considers that food and water intake increase with weight, and that a given degree of growth activity for the growing parts of the body must result in a larger absolute gain in a large rat than in a small rat. Now let us see how  $\ln \frac{A}{W}$  changes with a given percentage change in weight. It is obvious that

$$d \ln \frac{A}{W} = -d \ln W = -\frac{dW}{W}$$

Thus a given percentage increase in  $W$ , represented by  $\frac{dW}{W}$ , produces the same change in  $\ln \frac{A}{W}$  always, at all values of  $W$ , and this function therefore correctly

evaluates the effect of a change in weight upon the growth status. It is also obvious that

$$d \ln (A - W) = -\frac{W}{A - W} \frac{dW}{W}$$

A given percentage difference in  $W$  registers a difference in  $\ln (A - W)$  which increases rapidly as  $W$  approaches  $A$  (because of the  $A - W$  term, which approaches 0, in the denominator). Thus a small percentage increment in  $W$  which is of the same biological significance at all values of  $W$ , is incorrectly estimated by the function  $\ln (A - W)$  to be very much more important at values of  $W$  near  $A$  than at values far from  $A$ . Assuming an  $A$  of 270 gm., a 1 gm. change in a rat weighing approximately 240 gm. should according to this function represent the same change in growth status of the animal as an 8 gm. change in a rat weighing approximately 30 gm. As a rat approaches the limit weight the normal fluctuations around the equilibrium weight determined for that time by its inherent growth curve should be limited to fractions of a gram and finally approach 0. Yet the weight of such an animal near its limit weight is just as much determined by a dynamic equilibrium between processes causing gain in weight and processes causing loss in weight as in the rapidly growing animal; it is not reasonable to expect these processes later to become so much more finely balanced in their operation.

All the other  $A - W$  functions behave in similar fashion.

$$d \ln \frac{W}{A - W} = \frac{A}{A - W} \frac{dW}{W}$$

$$d \ln \frac{B + W}{A - W} = \frac{W(A + B)}{(B + W)(A - W)} \frac{dW}{W}$$

$$d \ln (A^{1/3} - W^{1/3}) = \frac{-\frac{1}{3}W^{1/3}}{A^{1/3} - W^{1/3}} \frac{dW}{W}$$

Each of them has a term in the denominator which approaches 0 as  $W$  increases. We bring these points up particularly because Brody *et al.* (1937) proposed an alternative law of relative growth

$$\ln (A_1 - W_1) = K \ln (A_2 - W_2) + \ln C$$

and Robertson (1907-08) long ago proposed the law

$$\ln \frac{W_1}{A_1 - W_1} = K \ln \frac{W_2}{A_2 - W_2} + \ln C$$

which are suggested by their respective time growth equations. Aside from the difficulty in interpreting the  $\ln C$  terms in these equations the terms  $\ln (A - W)$  and  $\ln \frac{W}{A - W}$  which are intended to put the different bodily parts

on a comparable basis independent of their various and widely different sizes, are not suitable measures of growth status. It is interesting to note that Brody, whose time growth equation contains the  $A - W$  term, and whose derivation definitely interprets  $A - W$  as a measure of growth status, nevertheless consistently prefers the function  $W/A$  as a practical measure of growth status in comparing growth curves.

*The Time Scale.*—In our growth formulation the time scale for pre-weaning growth (which like embryonic growth involves extensive histo-differentiation) is referred to conception, and the time scale for post-weaning growth (which is principally increase in size) is referred to birth. These reference points were chosen so as to get the best fit, and are therefore in a sense extra adjustable constants. However, the reference points once chosen are consistently adhered to for both sexes and all the different rat colonies fitted, so that they are not adjustable constants in the sense that  $k'$  and  $A$  are.<sup>2</sup> A rational approach to a reference point for the time scale in growth is a very difficult problem. The most common practice has been to take conception as the zero point, but most growth equations contain an adjustable constant which effectually shifts the origin to some other point (see  $t^*$  and  $T^*$  in Table I). And MacDowell has raised serious objection to the general assumption that conception ought to be the rational reference point:

"In mammals, the separation of the egg from the main food supply is correlated with a precocious development of the trophoblastic elements of the morula. These form the yolk sac and the traeger, which effect the first connection with the maternal food supply. They are well developed before the visible organization of the embryo is started. This preliminary stage occupies an appreciable portion of the gestation period. If natal and prenatal growth are to be compared . . . the growth curve of the embryo should start with the embryo proper, and not include the preliminary

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<sup>2</sup> It may be noted in passing that if a fully adjustable reference point for the time scale is introduced into the log reciprocal equation it becomes much more flexible, and is able to fit a great many data which the restricted equation cannot fit: the Donaldson rat data and most other available data on growth from a little before their points of inflection. But these data do not meet any reasonable nutritional standards, and in most cases there is no justification for dividing the data into two parts at the point where the three constant equation first begins to fit. Since the data are in all probability faulty, and since we have every reason to believe that nutritionally inhibited growth is very complex, the ability of the three constant equation to fit seems to be a positive disadvantage. The two constant equation is to be preferred in that it fits data expected to be simple, and data expected to be complex deviate from it in the manner to be expected. Furthermore while the reference point for time might differ in different species with different developmental histories, it is unlikely that it would properly differ for two sets of albino rats, yet this is what the three constant equation indicates for our data and the Donaldson data.

stage or the extraembryonic tissues . . . . As a practical criterion we propose the primitive streak stage" (MacDowell *et al.*, 1927).

We have gone even further and look upon the whole embryonic and pre-weaning growth period as a continuous series of changes in form and organization, with the characteristic structural pattern of the rat being approximately established only at the end of the suckling period; the primitive streak is only one of many stages in the series. It seems sufficiently rational that the reference point of this period should after all be conception, where this series of changes is initiated, and in our rats on good diets an excellent fit is obtained using conception as the reference point. We must then have a second reference point at a later age for the process of increase in size of the structural pattern (auxano-differentiation of Huxley) which was not in existence at conception. We are unable, however, to suggest a reason for placing this reference point at birth rather than at some other time beyond the general fact that birth is an important event in the life of the organism. As we have already pointed out, the one obvious special point in the period of auxano-differentiation is the end towards which growth is directed—the attainment of the final definitive body structure and size—and at this point the reciprocal time scale has its zero point.

*The Backman Equation.*<sup>3</sup>—In its general form this equation is too difficult to fit to be of much practical usefulness, but if the special case with  $s_2 = 0$  should prove to be generally applicable to the second phase of growth it would

<sup>3</sup> On re-reading Backman's papers we find that we had overlooked a rather important point. In Backman (1931) two equations are mentioned—equation 1 of Table I above and another equation, which we can call equation 3,

$$\frac{d \ln W}{dT} = e^{s_0 + s_1 \ln T + s_2 \ln^2 T}$$

both referred to as "meine Formel" and used interchangeably as though they were the same equation. The discussion at the end of the 1931 paper of the mathematical properties of the Backman equation refers to equation 1, while some of the data are fitted to equation 1 and some to equation 3. The very clear and straightforward presentation of "die Backman'sche Formel" in the two papers in 1938 refers entirely to equation 1 and no further mention is made of equation 3. Relying principally on these papers we missed the other equation, which is unfortunate because our log reciprocal equation is a special case of it, with  $s_0$  the same as our  $\ln k'$ ,  $s_1$  equal to  $-2$ ,  $s_2$  equal to zero. In a sense therefore the log reciprocal equation is not new, although as such it has not before been described by Backman or others. We have tried out this equation 3 on our data and find that it is no more successful than equation 1 in fitting the entire post-natal period of growth. The additional terms and adjustable constants do not improve the fit to the post-weaning data over that for the log reciprocal equation.

have much to recommend it. One feature is its parallelism in form with the log log equation which has been found to fit embryonic and pre-weaning growth so satisfactorily.

$$\ln W = K' \ln T + \ln C$$

$$\ln (A - W) = K \ln T + \ln C$$

Another feature which may or may not be valuable is the fact that a good fit results when the reference point for time is placed at conception. We prefer the log reciprocal equation on the basis of present knowledge because of its greater simplicity—it has one less adjustable constant—its greater ease of application, its relation with heterogony, the useful growth function  $\ln \frac{A}{W}$  and the interesting behavior of its time scale in becoming zero at the logical reference point for the auxano-differentiation process.

#### SUMMARY

The equation

$$\ln W = -\frac{k'}{t} + \ln A$$

fits the growth of well nourished rats from weaning on. The general properties of the equation, and particularly its relation with the law of relative growth, are discussed.

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## PURIFICATION AND CRYSTALLIZATION OF DIPHTHERIA ANTITOXIN

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The results of a series of investigations of the chemical nature of antibodies and antitoxins (Avery, 1915), (Gay and Chickering, 1915), (Felton, 1932), (Chow and Goebel, 1935), (Chow and Wu, 1937), (Kirk and Sumner, 1931, 1934), (Heidelberger and Kendall, 1936), (Pope, 1938), (Pope and Healey, 1939), (Petermann and Pappenheimer, 1941) have shown that these substances are proteins closely related to the normal serum proteins. Since they possess special properties not exhibited by the normal serum proteins they must have some special chemical structure but the nature of this structure still remains entirely unknown. The first step in an attempt to determine the structure is the isolation in pure form of the antitoxin. Chow, Goebel, Heidelberger, and their coworkers have obtained pneumococcus antibodies which were pure in the sense that they were completely precipitated by the specific carbohydrate. Petermann and Pappenheimer (1941) have isolated diphtheria antitoxin which was homogeneous by electrophoresis and ultracentrifuge but was not completely precipitated by diphtheria toxin.

Many antibodies form precipitates with their antigens and dissociation of this precipitate has frequently been used as a method of purification. Ramon (1922) found that diphtheria toxin precipitates when mixed in certain proportions with diphtheria antitoxin and was able (1923) to obtain some antitoxin by dissociating the complex in slightly acid solution. Pope and Healey (1939) and Petermann and Pappenheimer (1941) dissociated the complex by digestion with pepsin<sup>1</sup> at pH 3.0.

Pepsin attacks the antitoxin (Parfentjev, 1937) as well as the toxin and it seemed possible that a more homogeneous preparation might be obtained by using trypsin which, so far as is known, attacks only the toxin. If trypsin is added to a solution of the toxin-antitoxin complex in dilute acid and the solution then neutralized the toxin is destroyed and 30-60 per cent of the original antibody may be recovered. The antibody recovered in this way is 90 per cent or more precipitated by toxin; it is homogeneous in the ultracentrifuge or

<sup>1</sup> Preliminary experiments indicate that tetanus antitoxin may be purified in this way.

in the electrophoresis cell. The molecular weight of this antibody is 90,500 (Rothen, 1941) while antibody which has not been treated with trypsin has a higher molecular weight. Trypsin, therefore, digests part of the antitoxin as well as the toxin just as does pepsin (Petermann and Pappenheimer, 1941). This preparation corresponds closely to that obtained by Petermann and Pappenheimer. The solubility of this preparation is not constant, however, but varies with the amount of solid showing that at least two proteins are present, both of which react with toxin. The preparation may be further purified by fractional precipitation with ammonium sulfate. The most soluble fraction has constant solubility and appears to be a pure protein. It crystallizes readily in poorly formed thin plates. This protein is 90 per cent or more precipitated by diphtheria antitoxin and has about 700–900 antitoxin units per milligram protein nitrogen by the flocculation test and about 700 units per milligram by the animal protection test.

The preparation is homogeneous by electrophoresis or ultracentrifuge (Rothen, 1941). The protein is quite unstable and changes rapidly into a slightly less soluble form which does not crystallize and has a longer flocculation time but which has about the same antitoxic titer. This form is stable between pH 3.0 and 9.0. The carbohydrate content, calculated as glucose, is about 3 per cent.

Precipitation of purified antitoxin and crude toxin takes place over a wider range of concentrations than does the precipitation of crude antitoxin. Precipitation of pure antitoxin with purified toxin takes place over a very wide range and the soluble zone in the region of toxin excess disappears.

The toxin-antitoxin complex is soluble in the absence of salt and in solutions more alkaline than 7.0. This solution is precipitated by positive ions. The concentration of ions required to precipitate decreases as the valence increases. The reaction of toxin plus antitoxin, therefore, consists of two steps, as does the agglutination of bacteria. The first step is the formation of a compound. This is the specific part of the reaction. The precipitation of this compound then depends upon the salt concentration and hydrogen ion in the same way as does the precipitation of other colloidal suspensions.

The purified antitoxin does not precipitate with serum of rabbits immunized against normal horse serum. Guinea pigs sensitized with purified antibody react when injected with very small amounts of antibody but require very large amounts of normal serum to cause shock.

### *Experimental Results*

*1. Action of Trypsin on Toxin and Antitoxin.*—Diphtheria toxin is rapidly digested by trypsin whereas antitoxin is not. If trypsin were added to a solution of toxin-antitoxin, therefore, it would be expected that the toxin would be digested and that the complex would then dissociate until all the toxin was destroyed. This does not occur but instead there is a slow formation of non-protein nitrogen with no

TABLE I

*Digestion of Toxin-Antitoxin with Trypsin at pH 8.0*

100 ml. antidiphtheria plasma (500  $L_f$ /ml.) mixed with 1 liter diphtheria toxin (50  $L_f$ /ml.). Stood 25°C. 24 hrs., decanted. Precipitate suspension centrifuged and precipitate washed three times with  $m/20$  pH 7.4 phosphate buffer. Washed twice with water. Precipitate stirred with 100 ml. water. Gelatinous mass dissolved slowly to slightly opalescent solution. Titrated to pH 8.0 with  $NH_4OH$ . 0.05 mg. trypsin N/ml. added. Stood at 25°C. Analyzed for non-protein nitrogen. 5 ml. + 0.5 ml.  $m/2$  pH 7.4 phosphate. Stood 25°C. for  $\frac{1}{2}$  hour and filtered. Filtrate analyzed for antitoxin.

Time at 25°C.	Protein nitrogen/ml.	Non-protein nitrogen/ml.	$L_f$ /ml. filtrate after precipitation at pH 7.4
	mg.	mg.	
0	0.96	0.06	0
18 hrs.	0.70	0.30	<40
Same solution, boiled before addition of trypsin			
0	0.90		
10 min.	0.40		
1 hr.	0.30		
24 hrs.	0.20		

TABLE II

*Toxin-Antitoxin + Acetic Acid pH 4.0 (No Trypsin)*

	No.	Vol.	PN/ml.	$L_f$ /ml.	Total	$L_f$ /PN
			mg.			
200 ml. antidiphtheria plasms (500 $L_f$ /ml.) + 2000 ml. toxin (45 $L_f$ /ml.) at 25°C. for 24 hrs. Decanted. Precipitate suspension + 10 gm. Hyflo. Filtered and washed two times with $m/10$ pH 7.4 phosphate, washed two times with water. Precipitate suspended in 300 ml. water + 13 ml. 2 N acetic acid and filtered. Filtrate clear, pH 4.0-4.1 (brom cresol green) 24 hrs., 5°C.....	1	200	1.0	(450)	(90,000)	
50°C. 20 hrs. + 200 ml. water + 6 ml. 5 N $NH_3$ (pH 7.2), precipitate + Hyflo. Filtered. Filtrate.....	2	400	0.04	30	12,000	700
400 ml. No. 2 + 150 ml. saturated ammonium sulfate. Precipitate. Stood 25°C. + 3 gm. Hyflo. Filtered. Precipitate + $n/10$ pH 7.4 phosphate.....	3	50		200	10,000	
50 ml. No. 3 + 20 ml. saturated ammonium sulfate (0.35 saturated) slight precipitate + Hyflo. Filtered. Precipitate + phosphate.....	4	10	$\begin{cases} 0.26 \\ 0.32 \end{cases}$	$\begin{cases} 200 \\ 150 \end{cases}$	2,000	500
Filtrate.....	5	70	0.16	150	10,000	900

measurable liberation of antitoxin (Table I). If the toxin-antitoxin solution is boiled before the addition of trypsin it is rapidly digested. These results show that the toxin-antitoxin is not a denatured protein and suggest that the complex does not dissociate at pH 7.0-8.0.

Pappenheimer has shown that diphtheria toxin is unstable in acid and is rapidly denatured on the acid side of pH 6.0 and this observation has been confirmed in the course of this work. The toxin-antitoxin complex dissolves on the acid side

TABLE III

*Digestion of Toxin-Antitoxin from Crude or Purified Antitoxin at pH 3.5 and 7.4, 25°C.*

Toxin-antitoxin from plasma. Prepared same as in Table II except not washed with distilled water. Suspended in N/10 pH 7.4 phosphate. 0.30 mg. P.N./ml.

Toxin-antitoxin from purified antibody. 5000 *L*<sub>1</sub> units toxin mixed with 5000 units purified antitoxin. Precipitate decanted and washed with N/10 pH 7.4 phosphate. Suspended in N/20 pH 7.4 phosphate. Total N/ml. 0.16 mg.

20 ml. of suspension in N/10 pH 7.4 phosphate buffer titrated to pH 3.7 + N/10 hydrochloric acid. 0.05 mg. trypsin N/ml. added. Analyzed for non-protein nitrogen. Titrated to pH 7.2, stood 2-7 hrs. Filtered. Filtrate analyzed for non-protein nitrogen and antitoxin.

Time	pH	Toxin-antitoxin from plasma and toxin				Toxin-antitoxin from purified antibody			
		Total N/ml.	Non-protein N/ml.	P.N./ml.	L <sub>f</sub> /ml.	Total N/ml.	Non-protein N/ml.	P.N./ml.	L <sub>f</sub> /ml.
		mg.	mg.	mg.		mg.	mg.	mg.	
hrs.					(200 equivalent)				(200 equivalent)
0	3.7	0.38	0.05	0.33		0.16	0.06	0.10	
2.5	3.7		0.08	0.30			0.06	0.10	
		Titrated to pH 7.2							
0	7.2		0.08	0.30			0.06	0.10	
1	7.2		0.20	0.18			0.065	0.095	
3	7.2		0.19	0.19			0.075	0.085	
		Heavy precipitate. Filtered. Filtrate				Very slight precipitate. Filtered. Filtrate			
	7.2	0.26	0.19	0.07	80	0.16	0.07	0.09	80
		Filtrate from control solution, no trypsin				Control solution, no trypsin			
			0.05	0	0			0.04	40

of about pH 4.7 and if it were dissociated it would be expected that the toxin would be denatured and the antitoxin liberated. When crude toxin is precipitated with antitoxin and the entire suspension made acid and then alkaline, no precipitate appears, indicating that the toxin may have been destroyed. If the experiment is repeated with washed toxin-antitoxin in buffer, however, the precipitate reappears on neutralization. It is possible to recover some toxin by heating such an acid solution of toxin-antitoxin, as Ramon stated (Table II) but the yield is quite low. However, if trypsin is added to such an acid solution of toxin-antitoxin formed from either pure or crude antitoxin and the solution then neutralized, little or no pre-

cipitate occurs and the antitoxin is free in solution, whereas the toxin has disappeared.

Analysis of such solutions (Table III) shows that a rapid increase in non-protein nitrogen occurs as soon as the solution is brought back to pH 7.2. In the case of the toxin-antitoxin from crude antitoxin the increase amounts to nearly half the total protein nitrogen originally present while in the case of toxin-antitoxin from purified antitoxin the increase is much less. In both cases about one-third of the total antitoxin present is recovered free from toxin. This is similar to Pappenheimer's results with pepsin which appears to attack the antibody as well as the toxin. Since trypsin does not attack antitoxin alone nor toxin-antitoxin unless it is first acidified, the results indicate that the toxin-antitoxin complex is changed by acidification and that it is then hydrolyzed by trypsin in such a way as to destroy the toxin and also part of the antitoxin molecule.

If purified antibody, obtained by dissociating the toxin-antitoxin complex with acid alone (*cf.* Table II) is mixed with trypsin some of the protein is digested but there is also a loss of antitoxin. These results indicate that trypsin hydrolyzes the toxin-antitoxin complex in a different way from that in which it hydrolyzes either the toxin or antitoxin alone. The toxin-antitoxin complex acts in this respect like a distinct protein rather than a loose combination of toxin and antitoxin. This result confirms Heidelberger and Kendall's assumption that the toxin-antitoxin complex is a definite chemical compound. The reaction is peculiar in that a protein is left after the reaction is completed. This is also the case in the clotting of milk by either pepsin or chymotrypsin and also in the formation of pepsin, trypsin, or chymotrypsin from their precursors. All other proteolytic reactions, however, result in complete destruction of the protein.

#### *Effect of Varying Conditions on the Amount of Antitoxin Recovered*

The digestion of toxin-antitoxin just described is affected by the following variables: pH, time at each pH, concentration of salt, kind of salt, temperature, concentration of toxin-antitoxin, and concentration of trypsin. Obviously determination of strictly optimum conditions with such a large number of variables would involve an enormous number of experiments. Preliminary experiments showed that the time of standing in acid and also the pH of this acid solution made little difference so long as the toxin-antitoxin complex was dissolved (Table IV). Trypsin may be added before neutralization or immediately afterwards without affecting the yield. If the toxin-antitoxin complex is allowed to stand at pH 7.2 for several hours and trypsin then added, no antitoxin is recovered. The same results are obtained with toxin-antitoxin from either plasma or from purified antibody, except that in the latter case a small amount of antibody may be recovered without trypsin if the solution is allowed to stand for 20 hours or more in acid. The concentration of trypsin may be varied from 0.01 to 0.5 mg. per ml. without affecting the percentage of antibody recovered. The yield is the same with 0.5 M phosphate as with 0.05 M phosphate but if the solution is acidified with acetic acid and then neutralized with ammonia the yield is much less. The yield of antibody recovered without trypsin, however, is greater in acetic acid. The yield is the same at temperatures from 10–40°C. except that the reaction is slower at the lower temperatures.

The yield increases as the concentration of toxin-antitoxin decreases but below about 0.5 mg. protein nitrogen per ml. the increase in yield is small.

An outline of the method as finally worked out for handling large quantities of

TABLE IV

*Yield of Antitoxin Recovered after Various Times of Standing at pH 3.7 and pH 7.4 with or without Trypsin*

Antidiphtheria plasma mixed with equivalent volume of toxin, stood 24 hrs. 25°C., centrifuged. Precipitate washed two times with N/10 pH 7.4 phosphate. Suspended in N/10 pH 7.4 phosphate, volume equal to that of plasma. 25 ml. + 25 ml. N/10 hydrochloric acid (pH 3.7) 2 hrs. 5°C. as noted. 5 ml. + 1 ml. N/2 pH 7.4 phosphate. Stood at 25°C. 0.05 mg. crystalline trypsin nitrogen per ml. added as noted.

Toxin-antitoxin from antidiphtheria plasma				
Time at pH 3.7-4.0		Time at pH 7.2-7.6		Per cent original antitoxin recovered
hours		hours		
Without trypsin	With trypsin	Without trypsin	With trypsin	
0	0	0	24	0
0.01	0	0	4	25
0.0	0.01	0	4	30-50
1	0	0	4	30-50
0	1	0	4	50
7	0	0	4	35-50
0	7	0	4	50-70
24	0	0	4	35-50
0	24	0	4	50-70
0	24	0	24	50-70
1	0	0.15	24	25
1	0	0.50	24	30
1	0	4	24	0
1	0	24	24	0
24	0	24	0	10
Toxin-antitoxin from purified antibody				
0.01	0	0	24	50-60
0.5	0	0	24	50-60
20	0	0	24	50-60
20	0	24	0	15
0	0	4	4	<10

material is shown in Table V. All filtrations were carried out with suction using No. 3 Whatman paper and large Buchner funnels so that the final filter cake is not more than a few millimeters thick. If smaller filters are used the filtration may be slow.

Six different lots of antidiphtheria plasma and toxin have been used in the course of the present experiments. The results in general have been the same but the per cent of the original antitoxin recovered after treatment with trypsin varied from

20–60 per cent. It is not possible to state at present whether this variation is accidental or is caused by differences in the toxin or antitoxin preparation used.

The precipitate (3P) which forms after neutralization consists partly of unchanged toxin-antitoxin and a further 20–40 per cent antitoxin may be recovered by treating this precipitate as described for the original toxin-antitoxin suspension.

Filtrate No. 3 from this precipitate contains 20–60 per cent of the original antibody and has a titer of 300–400  $L_r$ /mg. protein nitrogen. The protein in this solution is 90 per cent or more precipitated by toxin. The solubility of the protein in this preparation, however, varies markedly with the quantity of solid present; in fact, about as much as does the solubility of serum globulin.

The protein may be further fractionated by precipitation with ammonium sulfate (Table V).

Most of the antibody is soluble in 0.33 saturated ammonium sulfate but precipitates at 0.5 saturated ammonium sulfate, as found by Pappenheimer and by Pope for antibody prepared by pepsin digestion. The fraction soluble between 0.33 and 0.60 saturated ammonium sulfate has a titer of from 500–700 flocculation units milligram protein nitrogen, or by the animal protection test. (The writer is indebted to Dr. W. E. Bunney at E. R. Squibb and Sons, New Brunswick, N. J., for carrying out this determination.) It is strictly homogeneous in the ultracentrifuge with a sedimentation constant of  $5.7 \times 10^{-13}$ . The diffusion constant is  $5.50 \times 10^{-7}$  cm.<sup>2</sup>/sec.<sup>-1</sup> and the molecular weight is 90,500 (Rothen, 1941). The material shows only one boundary in the electrophoresis cell at pII 7.3 or 3.0. (In dilute phosphate or veronal buffer there is some reversible spreading but measurements of electroendosmosis of this buffer in the microscopic cataphoresis cell (Northrop and Kunitz, 1925) show marked flow of water at the glass liquid interface. Addition of  $M/20$  calcium chloride, as is to be expected, completely prevents the electroendosmosis and also abolishes the reversible spreading.) This fraction probably corresponds closely to the preparation obtained by Pappenheimer and by Pope.

The solubility of this fraction, however, still varies markedly with the amount of solid (Fig. 1, second curve) showing that more than one protein is present in spite of the fact that it is homogeneous by ultracentrifuge and electrophoresis. This result is simply another example of the fact that the solubility method, which is theoretically identical with the classical melting point method, will distinguish between closely related proteins which are indistinguishable by other methods. Thus Landsteiner and Heidelberger (1923) found that hemoglobin from donkey and horse could be distinguished by this method although serologically they are extremely similar. Crystalline pepsin (Herriott, Desreux, and Northrop, 1940) prepared without special purification, is strictly homogeneous by electrophoresis or ultracentrifugation but does not have constant solubility and may be shown to contain several different proteins. Mixtures of samples of chymotrypsinogen of different solubilities are indistinguishable by electrophoresis (Butler, 1940) as are mixtures of pepsin and iodinated pepsin (Herriott, 1941) although in the latter case the solubilities are very different. Egg albumin from closely related species is also indistinguishable by the electrophoresis technique although they may be distinguished by serological tests (Landsteiner, Longworth, and van der Scheer, 1938).

Since the solubility of the solid in a dilute suspension is different from that in a



TABLE V  
Preparation of Pure Diphtheria Antitoxin

	No.	Vol.	PN ml. mg.	$\frac{L_f}{\text{ml.}}$	Total $L_f$	$\frac{L_f}{\text{mg. PN}}$
33 liters diphtheria toxin + 4 liters antidiphtheria plasma, 25°C. 24 hrs.....	1	37 liters	3	60	$2 \times 10^5$	20
Siphon off supernatant, precipitate suspension + 25 gm. Hyflo per liter, filter and wash precipitate 3 times with 1 liter M/10 pH 7.4 phosphate. Precipitate stirred + 5 liters 0.05 M $\text{KH}_2\text{PO}_4$ , titrate to pH 3.5, + 2.5 gm. crystalline trypsin. Stand 25°C. 24 hrs. Filter. Titrate filtrate to pH 7.4, cloudy.....	2	6.3 liters	0.3	100	$6 \times 10^5$	350
No. 2 + 700 ml. saturated ammonium sulfate (0.1 saturated) + 60 gm. Hyflo, filter. Filtrate clear.....	3	6.8 liters	0.17	70	$5 \times 10^5$	400
Precipitate.....	3P					
No. 3 + 2.3 liters saturated ammonium sulfate (0.33 saturated) + 50 gm. Hyflo. Filter. Precipitate (0.1-0.3 fraction).....	4				$1 \times 10^5$	350
Filtrate.....	5	8.6 liters	0.10	$\begin{cases} 60 \\ 50 \end{cases}$	$4 \times 10^5$	500
No. 5 + 2.1 liters saturated ammonium sulfate (0.45 saturated) 2 hrs. 25°C. + 50 gm. Hyflo. Filter. Precipitate (0.33-0.45 fraction).....	6				$2 \times 10^5$	450
Filtrate.....	7	11 liters	0.03	20	$2 \times 10^5$	700
No. 7 + 1500 gm. ammonium sulfate (0.65 saturated) 10°C. Settle. Decant supernatant. Precipitate suspension + 20 gm. Filter-cel. Filter. Precipitate.....	8				$2 \times 10^5$	700
Precipitate 8, stir + 3 liters (0.5 saturated ammonium sulfate, 0.05 M $\text{PO}_4$ pH 7.4), filter. Filtrate.....	9	3 liters	0.07	$\begin{cases} 45 \\ 50 \end{cases}$	$1.5 \times 10^5$	$\begin{cases} 650 \\ 700 \end{cases}$
3 liters No. 9 + 60 ml. (2.5 N $\text{H}_2\text{SO}_4$ , 0.5 saturated ammonium sulfate) 25°C. 1 hr. + 20 gm. Filter-cel. Filter. Filtrate.....	10	3 liters	0.05	$\begin{cases} 30 \\ 35 \end{cases}$	$1 \times 10^5$	$\begin{cases} 600 \\ 700 \end{cases}$

TABLE V—*Concluded*

	No.	Vol.	PN ml. mg.	$L_f$ ml	Total $L_f$	$L_f$ mg PN
Titrate No. 10 to pH 6.8, stir in saturated ammonium sulfate slowly until silky precipitate forms (about 200 ml. saturated ammonium sulfate). Stand 6°C. 24 hrs. Decant and centrifuge. Precipitate suspension, needles, plates and some amorphous. Suspend in 50 ml. 0.5 saturated ammonium sulfate	11	50 ml.	1.0	$\begin{Bmatrix} 650 \\ 700 \end{Bmatrix}$	$3 \times 10^4$	$\begin{Bmatrix} 650 \\ 700 \end{Bmatrix}$
No. 11 + 10 ml. water (clear) + saturated ammonium sulfate till slightly turbid. Stand 25°C. 2 hrs., thin plates, no amorphous. Stand 25°C. 24 hrs., centrifuge. Precipitate + 20 ml. 0.5 saturated ammonium sulfate. Precipitate suspension	12	20 ml.	1.0	$\begin{Bmatrix} 600 \\ 700 \end{Bmatrix}$	$1.2 \times 10^4$	$\begin{Bmatrix} 600 \\ 700 \end{Bmatrix}$
No. 12 + 5 ml. water + saturated ammonium sulfate slowly until slightly turbid. Stand 25°C. 1 hr., heavy precipitate thin plates. Centrifuge. Dissolve precipitate + 10 ml. water.	13	10 ml.	1.0	$\begin{Bmatrix} 650 \\ 700 \end{Bmatrix}$	$6 \times 10^3$	$\begin{Bmatrix} 650 \\ 700 \end{Bmatrix}$

concentrated one, the composition of this solid must be different and hence a partial separation has been effected. On paper it is only necessary, therefore, to repeat the solubility experiment until a pure protein of constant solubility is obtained.<sup>2</sup> Actually, however, this cannot be done since the yield is small and the substance too unstable. The fraction soluble in 0.5 saturated ammonium sulfate but not at 0.65 has more nearly constant solubility, indicating that the more soluble fraction could be more easily purified than the less soluble. A very small amount of a protein having

<sup>2</sup> If the protein is a solid solution of two proteins and obeys Raoult's law then the maximum change in composition which can be obtained in one step is from  $F$  to  $CF$  when  $F$  is the original ratio of the two constituents and  $C$  is the ratio of their solubilities in the solvent used (Northrop and Kunitz, 1930). If a solvent could be found in which the solubility of one component was 0, then the separation would be complete in one step. For this reason it is to be expected that a better separation will be obtained in a solvent in which the total solubility is small and this is the case. A similar result was obtained in the case of pepsin (Herriott, Desreux, and Northrop, 1940).

constant solubility (bottom curve, Fig. 1) was eventually obtained by extracting with one-half saturated ammonium sulfate followed by precipitation at pH 3.5 as shown in the table. This protein is homogeneous by electrophoresis and ultracentrifuge and fulfills the criteria for a pure protein.

Inspection of the solubility curves shows that only sample 48B12 had strictly constant solubility. The other samples tested all showed minor discrepancies al-

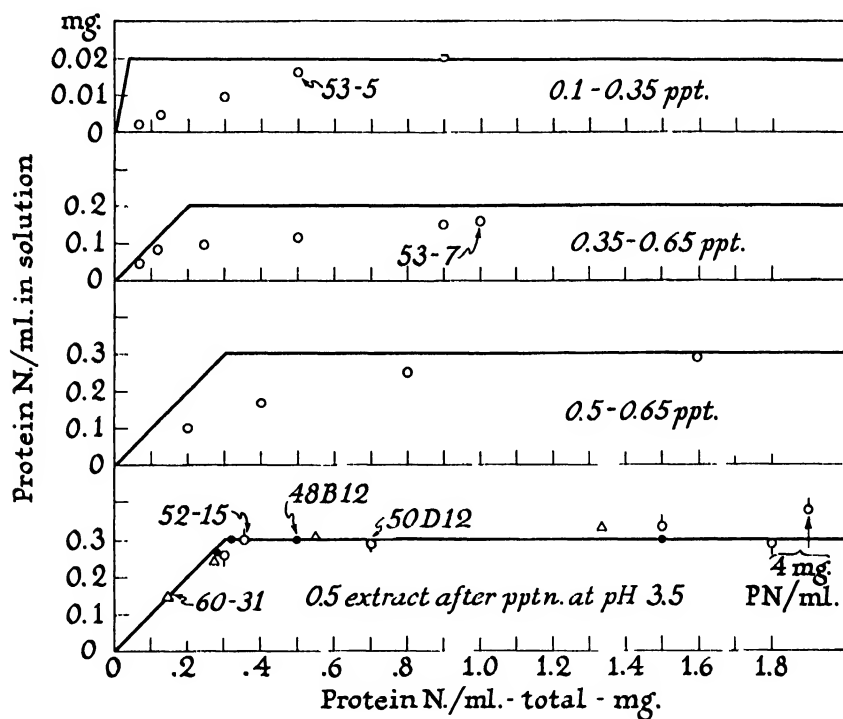


FIG. 1. Solubility curves of various antitoxin fractions. The protein nitrogen per milliliter in solution is plotted against the total protein nitrogen concentration. The solid lines are the theoretical curves for a solid phase of one component.

though the curves are better than those of other proteins with the exception of chymotrypsinogen, trypsin, and pepsin. The determinations were all made with the amorphous preparation since some decomposition occurs during crystallization and the crystals do not have as good a solubility curve. The samples tested in Fig. 2 were analyzed within a few hours after their preparation.

Solutions of the protein crystallize readily in the form of thin, more or less irregular plates (Fig. 2). These plates resemble very closely the first crystals of ribonuclease isolated by Kunitz (1940). On longer standing or on recrystallization the ribonuclease crystals become beautifully regular, whereas the antibody crystals remain about

the same.<sup>3</sup> The first crystals which appear are the best and after a few hours crystallization stops. On recrystallization the same process is repeated. Crystals form rapidly and are as good as would be expected under the conditions but instead of improving on standing the crystallization again stops. The protein in the mother liquor cannot be crystallized and has a low solubility very much like that of the 0.5-0.6



FIG. 2. Crystals of diphtheria antitoxin.

fraction from which the pure protein was originally isolated (Fig. 3). At the same time, the time required for flocculation increases, when the antitoxin is mixed with

<sup>3</sup> Large well formed prisms have appeared in two preparations after 3-4 months' standing. These preparations had been sealed with vaseline under a cover slip on a slide. The original poorly formed plates disappear as the larger crystals develop indicating that the large crystals are either the antibody itself or a decomposition product. It has not been possible so far to prepare enough of these crystals to test for antibody content.

toxin, although the final titer remains the same (Table VI). The results show that the pure protein is very unstable and is rapidly transformed to a slightly less soluble form. The presence of this form prevents crystallization. Most protein solutions, if allowed to stand in strong salt solution, form thin plates on the surface which may settle subsequently and which resemble somewhat the antitoxin plates. These surface films differ, however, in that they are more irregular and are usually spotted with

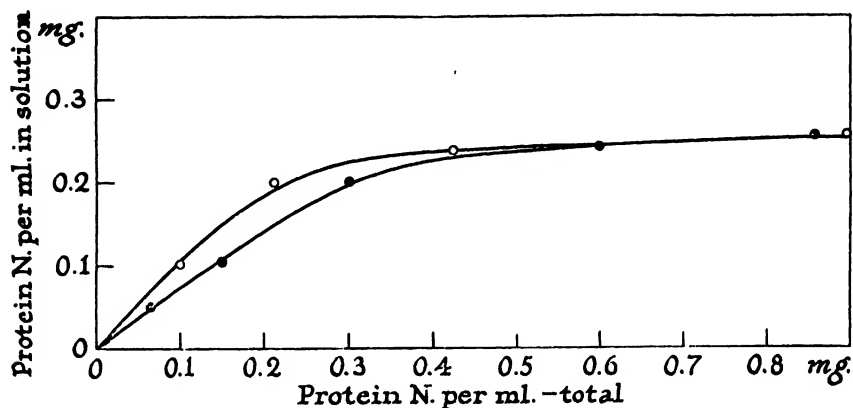


FIG. 3. Effect of standing in 0.4 saturated ammonium sulfate pH 7.0 on solubility of crystalline antitoxin in (0.5 saturated ammonium sulfate, 0.05 M pH 7.4 phosphate). Upper curve—solubility after 1 hour at 25°C. Lower curve—after 24 hours.

TABLE VI  
*Increase in Flocculation Time of Purified Antibody on Standing at 25°C.*

1 ml toxin (45 L <sub>f</sub> /ml) +	0.2	0.25	0.3	0.4 ml 87-1 (0.5 saturated ammonium sulfate extract)
Time at 25°C	Time for flocculation 50°C			
days	min	min.	min.	min
0		10	10	12
1	45	15-18	15-18	20
2	40	17	15	17
4		20	14	15

irregular markings. In addition the formation of the antitoxin plates is greatly accelerated by inoculation of the supersaturated solution while the formation of surface plates is not. Under these conditions crystallization may be practically complete in  $\frac{1}{2}$  to 1 hour and it can be seen that the plates are formed in the body of the solution and not on the surface. The plates are faintly doubly refractile when observed with the Nicol prism and analyzer. An intense light is necessary as, owing to the thinness of the plates, the double refraction is slight. The straight lines in the photograph are not needles, but the edges of the plates. This is clearly evident under the microscope.

The plates are slightly less soluble than the amorphous form although the difference is much less than is the case with pepsin.

These results indicate that the plates from saturated solution of this protein are true crystals although final decision depends on the results of x-ray analysis.

TABLE VII

*Per Cent Protein Nitrogen Precipitated from Various Samples of Antitoxin by Crude or Purified Toxin*

5 ml. crude or purified toxin solution added to various samples of antitoxin containing equivalent number of units in  $n/10$  pH 7.2 phosphate. Protein nitrogen per ml. determined. 50°C. 20 hrs. 25°C. 48 hrs. Centrifuged and protein nitrogen determined in supernatant (turbidity).

Sample No.. .. .	51A41	86-5	86B1	86-25	86A16	90-13
	Globulin fraction	0.1-0.3 sat. a.s. fraction	0-0.35 sat. a.s. fraction	0.4-0.5	crystals	(2 × ppt. + toxin)
$L_f$ /mg. P.N. . . . .	150	500	250	800	750	900
Crude toxin						
Total P.N., mg. . . . .	2.25	0.75	1.1	0.60	0.60	0.48
P.N. in supernatant, mg. . . . .	1.95	0.06	0.12	0.03	0.06	0.07
Per cent P.N. in supernatant. . .	86	8	10	5	10	14
Per cent P.N. in precipitate. . .	14	92	90	95	90	86
Purified toxin (Pappenheimer)						
Antitoxin P.N., mg. . . . .	2.0	0.72		0.48	0.50	0.50
Toxin P.N., mg. . . . .	0.11	0.22		0.22	0.22	0.14
Total P.N., mg. . . . .	2.11	0.94		0.70	0.72	0.64
P.N. in supernatant, mg. . . . .	1.97	0.04		0.048	0.08	0.03
Per cent total P.N. in supernatant. . . . .	93	4		7	11	4
Per cent P.N. in precipitate. . .	7	96		93	89	96

*Precipitation of Various Fractions of Purified Antitoxin by Crude and Purified Toxin*

Mixtures of diphtheria toxin and antitoxin precipitate over a rather narrow range of concentrations when allowed to stand. Preliminary experiments showed that precipitation increased for 10 or 15 minutes at 50°C. but after 20 hours remained constant. The experiments shown in Table VII were made at the equivalent point; *i.e.*, with that proportion of toxin and antitoxin which first precipitates. The results with other mixtures near this range are not significantly different.

The first sample, 51A41, was a globulin fraction prepared directly from antidiph-

theria plasma without previous precipitation with toxin. It had an antitoxin titer of about 150 units per mg. protein nitrogen and only about 7 per cent of the total nitrogen was found in the precipitate. This figure, of course, is subject to a large error since it is determined by difference. The other preparations represent different fractions obtained by fractional precipitation with ammonium sulfate and have antitoxic titers varying from 250 units per mg. protein nitrogen to 900. However, as Table VII shows, 90 per cent or more of the total protein nitrogen is precipitated from all of these preparations. The table also shows that the amount of protein nitrogen left in the supernatant when crude toxin is used is about the same as when purified toxin is used. Evidently the protein in the crude toxin preparation used was practically all toxin protein.

It follows from these determinations that the various fractions must combine with different quantities of toxin. 0.72 mg. protein nitrogen of 86-5, which is the fraction obtained between 0.1-0.3 ammonium sulfate precipitated 0.22 mg. of toxin protein nitrogen, a ratio of about 3 to 1, whereas the preparations having a higher antitoxic titer precipitate an amount of toxin protein equivalent to about one-half the antitoxin. These figures are somewhat uncertain owing to the fact that some protein nitrogen is not precipitated and it is not possible to say whether this is antitoxin protein or toxin protein or partly both. The precipitate has no measurable solubility in M/20 phosphate buffer and it seems unlikely that the protein remaining in solution was really due to the solubility of the precipitate. Since the precipitate appears to be completely insoluble it follows that the quantity formed will be independent of the volume and this is the case as Heidelberger and Kendall (1935) have previously found. However, this would be true of any insoluble precipitate and it does not seem possible to draw any conclusions from this result concerning the composition of the precipitate.

Kekwick and Record (1941) have obtained partially purified antitoxic preparations by means of electrophoresis which also showed varied combining ratios with toxin and have suggested, therefore, that there are at least two antitoxins. The present experiments confirm this conclusion.

### *The Effect of the Purity of the Preparations on the Precipitation Zone*

The precipitation of crude diphtheria toxin with antitoxin is peculiar in that the precipitation range is narrow. For this reason the titration is accurate to about  $\pm 20$  per cent. The precipitation of crude or purified toxin solutions with different dilutions of antidiphtheria plasma or purified antibody is shown in Table VIII. The table shows that with crude toxin and antidiphtheria plasma only one tube shows complete precipitation; *i.e.*, either twice as much plasma or one-half as much plasma does not give a flocculent precipitate. When crude toxin is precipitated by purified antibody the range of precipitation is doubled and it requires a larger excess of antibody to prevent precipitation. For this reason the titration of purified antitoxin is much less accurate than the titration of antidiphtheria plasma. When purified toxin is mixed with purified antibody the range is extended so that complete precipitation occurs even in the presence of an excess of antibody which is equivalent to four times the toxic equivalent and extends far down into the region of excess toxin. These and the preceding experiments indicate that a possible cause for the varying composition of

toxin-antitoxin complexes is due to the fact that there are more than one toxin and more than one antitoxin.

The purified toxin used in these experiments was kindly supplied by Dr. A. M. Pappenheimer, Jr. It was homogeneous by electrophoresis or ultracentrifuge but may still have contained more than one protein since its solubility has not been tested (Petermann and Pappenheimer, 1941).

TABLE VIII

*Precipitation of Antidiphtheria Plasma or Purified Antibody with Crude or Purified Toxin*

1 ml. toxin solution containing 40  $L_I$ /ml. in N/10 pH 7.4 phosphate mixed with 1 ml. various antitoxin preparations diluted as noted with N/10 pH 7.4 phosphate and allowed to stand at 25°C.

Time at 25°C.	Dilution of plasma....	Amount of precipitation							
		1	1/2	1/4	1/8	1/16	1/32	1/64	1/168
hrs.		Crude toxin + crude antibody							
1.5		-	+	++	C	+	-	-	-
24		-	+	++	C	+	-	-	-
		Crude toxin + purified antibody 750 $L_I$ /mg. P.N.							
1.0		-	-	++	C	+	-	-	-
24		-	+	C	C	+	-	-	-
		Purified toxin + purified antibody							
1.0		+	+++	C	C	+	-	-	-
24		+	C	C	C	C	C	C	+++

*Effect of Salts on the Precipitation of Toxin-Antitoxin*

The formation of toxin-antitoxin precipitates is similar in many respects to the specific agglutination of bacteria. In the case of bacterial agglutination it has been repeatedly shown that (Bordet, Joos, Bechold, Porges, Porges and Prantschoff, Northrop and DeKruif (*cf.* Topley and Wilson, page 154)), the reaction takes place in two steps. The first step is the combination of antibody with the bacterial cell. This is the specific step in the reaction and, as Heidelberger and Kendall's (1935) results indicate, is very likely a chemical reaction. In the absence of electrolytes and especially in slightly alkaline solution no agglutination takes place. However, if electrolytes are now added to such sensitized suspensions, agglutination occurs and the effect of the electrolytes may be predicted from the effect of electrolytes upon colloidal suspensions in general. Such suspensions are characterized by the fact that they are agglutinated by ions having an opposite charge from the particles and that the agglutinating concentration decreases rapidly as the valence of the ion increases. This observation was originally made by Hardy and applies to the oil droplet (Powis, 1924), collodion particles coated with denatured egg albumin, or denatured proteins



in general (Loeb, 1924), or sensitized bacteria (Northrop and DeKruif, 1922). This valency effect of salts is quite distinct from the effect of salts on solutions of crystalloids or on the solubility of native proteins. It appears to be characteristic of antigen-antibody complexes that the solubility or stability of the complex is determined by the electrical charges as is the stability of colloidal suspensions in general, while the solubility of the antigen or antibody separately may be affected by electrolytes in an entirely different way and in the same way as are solutions of crystalloids or native proteins.

If toxin-antitoxin complex is formed from antidiphtheria plasma and crude toxin and then washed repeatedly with dilute phosphate buffer it remains completely in-

TABLE IX

*Precipitation of Solution of Toxin-Antitoxin in Water by Acid and Salts*

Sample 142-5 (7.5 mg. P.N./ml.) diluted 1/20 with water-clear—No. 1.

2 ml. No. 1 +	0	0.1	0.2	0.3	0.5	0.7	1.0 ml. N/4,000 acetic acid.
Precipitation	—	+	+	++	C	C	+
pH (brom cresol purple)	7.6	6.6	6.55	6.5	6.45	6.4	6.3

1 ml. No. 1 + 1 ml. various salt solutions pH 7.4 in N/50 pH 7.4 veronal buffer; dilutions made with water.

Salt	Final concentration salt												
	M/20	M/40	M/80	M/160	M/320	M/640	M/1280	M/2560	M/5120	M/10,000	M/20,000	M/40,000	M/80,000
	Precipitation after 20 hrs. 25°C.												
NaCl.....	C	C	+	—	—								
pH 7.4 veronal.....	C	++	+	—	—								
Na <sub>2</sub> SO <sub>4</sub> .....	C	C	++	+	—	—							
CaCl <sub>2</sub> .....	C	C	C	C	C	C	++	+	—				
LaCl <sub>3</sub> .....				C	C	C	C	C	C	C	C	C	++
													+

soluble. If this precipitate is then washed repeatedly with distilled water it becomes gelatinous and finally dissolves. The solution in distilled water alone at about pH 7.0 is more or less opalescent but at pH 8.0–9.0 it is quite clear and resembles a solution of denatured protein. If electrolytes or acid are now added to such a solution precipitation occurs and the concentration of electrolytes required decreases rapidly with the valency of the positive ion (Table IX). The results in general are exactly similar to those obtained with the oil droplets, etc. described above. In addition, acid causes precipitation over a narrow range of pH of about 6.4–6.5, the width of this range depending upon the concentration of the solution.

As in the case of bacteria, therefore, the toxin-antitoxin complex behaves in regard to salt precipitation like a preparation of denatured serum protein as Shibley (1926) has emphasized in connection with bacterial agglutination. In the case of the toxin-antitoxin complex, however, the protein is probably not denatured since the results of the experiments with trypsin digestion previously described (Table I) show that this

complex is not attacked by trypsin, whereas if it is boiled the complex is rapidly digested. Trypsin attacks native proteins slowly if at all so that the failure of trypsin to digest the toxin-antitoxin complex indicates that the protein is still native.

### *Stability of Purified Antitoxin*

The experiments described in connection with the crystalline antitoxin show that the strictly homogeneous protein is extremely unstable and changes very rapidly to a slightly less soluble form. After this preliminary change, however, the preparation is quite stable over a pH range of 2.0-8.5. Within this range little or no loss of ac-

TABLE X

#### *Inactivation of Purified Diphtheria Antitoxin at Various pH*

Purified antitoxin (0.4 saturated-0.5 saturated ammonium sulfate fraction) in  $N/10$  pH 7.4 phosphate and 0.05 saturated ammonium sulfate; P.N./ml. 0.5 mg. 5 ml. plus noted ml. sodium hydroxide or sulfuric acid. Stand 25°C. 1 ml. sample + 4 ml.  $N/2$  pH 7.4 phosphate and titrated against crude toxin.

Ml. 2 N sodium hydroxide Ml. 1 N sulfuric acid. . . . pH (colorimetric) . . . . .	1.0 <1.0	0.5 <1.0	0.25 1.5	0.1 3.6	0 7.4	0.2 8.0	0.5 8.5	1.0 9.0	2.0 9.8
Time at 25°C.	<i>Lf/ml. after standing at 25°C. as noted</i>								
<i>hrs.</i>									
0.25	{ 250 200	{ 350 300	400	400	400	400	400	{ 200 300	{ 200 150
24	50	100	200		{ 350 400	{ 350 400	{ 350 400	{ 200 250	150
Precipitation after 24 hrs.	+++	+++	+++	-	-	-	+	++	++

tivity is observed in 24 hours at 20°C. (Table X). In more alkaline or more acid solutions precipitation occurs and there is marked loss in antitoxic value.

### *Carbohydrate Content of the Antibody Preparations*

Petermann and Pappenheimer (1941) have found that the carbohydrate content of purified antibody prepared by digestion of the toxin-antitoxin complex by pepsin is higher than in crude antibody fractions prepared directly from immune serum. The carbohydrate content of the antibody fraction prepared by the present method does not appear to differ significantly from that of antitoxin prepared by fractionation direct from antisera (Table XI). Some variation was found in the carbohydrate content and one preparation, 62-2, gave 3.4 per cent. This variation in the carbohydrate content and the general character of the preparations suggests the possibility that a part, at least, of this carbohydrate, is derived from the serum mucoid and is not a part of the antitoxin molecule itself. However, all attempts to reduce the carbohydrate content below about 2 per cent have been unsuccessful.

*The Relation of the Purified Antibody to the Proteins of Normal Serum*

It has frequently been suggested that antibodies are formed by a slight modification of normal serum proteins. They are, however, extremely closely related to them as evidenced by the fact that some of Pappenheimer's preparations, which contain less than one-half antibody, were homogeneous by electrophoresis and ultracentrifuge,

TABLE XI  
*Glucose Content of Various Antitoxin Preparations*

Per cent glucose analyzed according to Sørensen and Haugaard (1933) compared with standard glucose solutions					
Sample.....	51A41 0.4-0.5 fraction from antidi- phtheria plasma	86-25 0.4-0.5 saturated ammonium sul- fate fraction from purified antitoxin	86A67 Purified toxin once crystal- lized	62-2 Purified toxin two times crystal- lized	90-15 Antitoxin precipi- tated two times with toxin
Per cent glucose.....	2.4	2.0 2.4	2.8	3.4	2.8

TABLE XII  
*Fractional Precipitation of Antidiphtheria Plasma or Normal Plasma and Pure Antitoxin with Varying Concentrations of Ammonium Sulfate*

Ammonium sulfate concentration	Antidiphtheria plasma			Normal plasma + purified antitoxin			Normal plasma
	Per cent P.N.	Per cent $L_f$	$L_f$ /P.N.	Per cent P.N.	Per cent $L_f$	$L_f$ /P.N.	Per cent P.N.
<i>per cent saturated</i>							
Original	(100)	(100)	30	(100)	(100)	10	
0-35	44	10	10	45	<10	<10	40
35-45	19	50	75	8	50	60	10
45-55	9	40	100	6	30	50	10
55-65	25	<10		30	<10		40

and also by the fact that the antibody can only be separated from the normal proteins by fractional precipitation with extreme difficulty.

If the purified antibody obtained by the action of trypsin were different from the form in which it occurs in the original immune plasma, it might be expected that the purified antibody could be separated from normal plasma more easily than the naturally occurring antibody can be separated from the proteins of immune plasma. In order to test this assumption antidiphtheria plasma containing about 500 antitoxic units per ml. and an "artificial" immune plasma containing the same number of anti-

toxic units prepared by adding purified antitoxin to normal plasma, were precipitated by varying concentrations of ammonium sulfate. The results of the experiment are shown in Table XII. It is evident that the antitoxin distributes itself in the same way in both cases since the percentage of the original antitoxin found in the various fractions is within the limits of error the same. The antitoxic titer per milligram of protein nitrogen of the various fractions is also about the same and it is just as difficult to concentrate the antitoxin from the "artificial" immune plasma as from natural immune plasma.

### *Immunological Relations of the Purified Antitoxin to Normal Serum*

Some of the immunological properties of the purified antitoxin have been determined by Dr. Carl TenBroeck. The serum of a rabbit immunized against normal horse serum gave a precipitate with 1/4,000 ml. normal horse serum (containing about 0.002 mg. protein nitrogen) but gave no precipitate with 1 ml. of a solution of purified antibody containing 1/10 mg. protein nitrogen.

Guinea pigs sensitized by the subcutaneous injection of 0.003 mg. of purified antibody nitrogen gave a typical anaphylactic reaction 3 weeks later when 0.05 mg. of antibody protein nitrogen was injected intravenously. Similarly sensitized guinea pigs failed to react to normal horse plasma diluted 1:10, but 4 out of 6 reacted to the normal undiluted plasma containing 0.5 mg. of protein nitrogen.

The purified antitoxin is, therefore, antigenically distinct from the normal serum proteins. The fact that guinea pigs sensitized with purified antibody react with large amounts of normal horse serum probably indicates the presence of minute amounts of normal protein in the purified antibody preparation. Extremely minute amounts are sufficient to sensitize an animal and it is quite possible that such minute amounts of normal protein could be carried through the various steps of purification.

### *Experimental Methods*

**Protein Nitrogen.**—1 ml. sample containing from 0.01–0.10 mg. protein nitrogen was added to 9 ml. (2.5 per cent trichloroacetic acid, 10 per cent saturated ammonium sulfate) and boiled. The suspension was allowed to cool and the turbidity measured against a standard suspension of purified antibody in a Klett photoelectric colorimeter. The standard suspension was analyzed by micro-Kjeldahl. The values for protein nitrogen in the purified samples are accurate to  $\pm 5$  per cent but those of the crude preparations and especially of the plasma fraction may be in error by 20–30 per cent since the turbidity factor varies with different proteins.

**Flocculation Test.**—Varying quantities of the solution to be tested were added to 1 ml. of toxin solution which had been titrated against a standard antibody preparation. The tubes were immersed in a 50°C. water bath and the end-point taken as that tube which first showed a flocculent precipitant. With antidiphtheria plasma this method is accurate to  $\pm 20$  per cent but with purified antibody the precipitation range is much wider and the error may be  $\pm 20$ –30 per cent.

**Solubility Determination.**—50–100 mg. of the sample were precipitated by 0.5 saturated ammonium sulfate, centrifuged, and the precipitate stirred with 5 ml. (0.5 saturated ammonium sulfate, 0.05 M pH 7.4 phosphate buffer) and centrifuged.

This process was repeated until the protein nitrogen per ml. of the supernatant was the same in two successive supernatants. The solid is now in equilibrium with this solution (*cf.* Butler, 1940). 0.1, 0.2, 0.5, 1.0, and 2.0 ml. of the suspension are placed in small centrifuge tubes and the volume of each made up to 3 ml. with the above solvent. The tubes are allowed to stand with occasional stirring for 2 hours. 0.1 ml. samples are removed for determination of total protein nitrogen, the tubes centrifuged, and the supernatant analyzed for protein nitrogen. It is possible to make a complete solubility curve with 6.0–10 mg. protein nitrogen and all of this may be recovered except for the amount actually used in the analytical samples.

The analyses reported were carried out by Miss Elizabeth Shears.

#### SUMMARY

Purified preparations of diphtheria antitoxin have been obtained by digestion of the toxin-antitoxin complex with trypsin, followed by fractional precipitation with ammonium sulfate. The various fractions obtained in this way are all 90 per cent or more precipitated by diphtheria toxin but combine with different quantities of the toxin.

The fraction precipitated between 0.33 and 0.5 saturated ammonium sulfate is homogeneous by electrophoresis and ultracentrifuge but does not have constant solubility.

A small amount of a more soluble fraction has been obtained which does have constant solubility and satisfies the criteria of a pure protein. This protein crystallizes readily in poorly formed thin plates. It is very unstable and reverts to a less soluble non-crystallizable form. It has a sedimentation constant of  $5.7 \times 10^{-13}$  and a molecular weight of 90,500. It has an antitoxic value of 700–900 flocculation units per mg. protein nitrogen and has an antitoxic value by the protection test of about 700 units per mg. protein nitrogen.

The precipitation range of the purified antitoxin with purified toxin is much wider than that with crude preparations.

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# PURIFIED DIPHTHERIA ANTITOXIN IN THE ULTRACENTRIFUGE AND IN THE ELECTROPHORESIS APPARATUS

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Several preparations of diphtheria antitoxin of different degrees of purity described by Northrop (1) in the preceding article were investigated in the ultracentrifuge and in the electrophoresis apparatus. The main object was to test the homogeneity of the materials and also to obtain a value for the molecular weight of the purified antitoxin.

*Ultracentrifuge Studies.*—At the time this investigation was started, the only ultracentrifuge studies reported on diphtheria antitoxin were those of Pappenheimer, Lundgren, and Williams (2) and of Paic (3). The molecular weight of antitoxin arrived at by Pappenheimer and coworkers was 184,000, whereas Paic, in a very indirect way, estimated that the size of antitoxin was between that of serum albumin and serum globulin. It soon became evident that the preparations obtained by Northrop with trypsin digestion were homogeneous in the ultracentrifuge, but that the value of the sedimentation constant,  $s_{20}^{\text{water}} \simeq 5.5 \times 10^{-13}$  was considerably smaller than the value  $s_{20}^{\text{water}} = 7.2 \times 10^{-13}$  found by Pappenheimer *et al.* Since then, Petermann and Pappenheimer (4) obtained by pepsin digestion an antitoxin fraction which was homogeneous in the ultracentrifuge, with a sedimentation constant  $s_{20}^{\text{water}} = 5.7 \times 10^{-13}$  but only 33 per cent of the material was specifically precipitated by toxin. Tiselius and Dahl (5) found also very recently that a pepsin-digested fraction, prepared according to Pope, was homogeneous in the ultracentrifuge with a constant of sedimentation  $s_{20}^{\text{water}} = 5.4$  to  $5.9 \times 10^{-13}$ . The results which were obtained in the present study are summarized in Table I.

The samples of antitoxin obtained by treatment of the toxin-antitoxin complex with trypsin had different antitoxin titer by the flocculation test. They were also completely precipitated by toxin (Northrop (1), Table VII). However, it is apparent from the table that they all had very nearly the same constant of sedimentation,  $s_{20}^{\text{water}} \simeq 5.5 \times 10^{-13}$ . The difference between the highest value, 5.7, and the lowest, 5.3, is probably not significant, especially if one considers that the value 5.3 was obtained in an experiment with a high protein concentration. The sedimentation constant for the crystalline material was  $s_{20}^{\text{water}} = 5.65 \times 10^{-13}$ . It seems probable that the preparations con-



TABLE I  
*Sedimentation Data*

Sample	Northrop corresponding sample	Concentration protein	Medium	pH	$\delta_{20}$	$\eta_{sp}$	$t$	$s_2 \times 10^{13}$	$s_{20}^0 \times 10^{13}$
		<i>per cent</i>				<i>milli-poise</i>	$^{\circ}\text{C.}$		
1. Trypsin-digested	Fract. 6, Table V	1.1	Phosphate 0.1 M	7.5	1.0070	11.60	27.2	6.00	5.27
2. Trypsin-digested	Fract. 8, Table V	0.5	Phosphate 0.05 M	7.35	1.0043	11.61	11.9	4.34	5.56
3. Trypsin-digested	Fract. 8, Table V	0.3	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0107	10.46	18.6	4.85	5.41
4. Trypsin-digested	Fract. 8, Table V	0.15	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0107	10.46	17.2	4.92	5.40
5. Trypsin-digested	Fract. 8, Table V	0.65	Phosphate 0.1 M plus 2 per cent $(\text{NH}_4)_2\text{SO}_4$	7.4	1.0258	11.48	1.63	4.38	5.73
6. Trypsin-digested	Fract. 12, Table V	0.2	Phosphate 0.05 M	7.35	1.0043	11.61	18.3	5.28	5.65
7. Trypsin-digested	Fract. 8, Table V	0.16	Acetate 0.1 M	2.9	1.0077	10.40	11.9	4.29	5.56
8. No enzymatic digestion	Fract. 45-55, Table XII	0.5	Phosphate 0.1 M plus 2 per cent $(\text{NH}_4)_2\text{SO}_4$	7.4	1.0390	11.70	24.5	5.75	6.79
9. No enzymatic digestion	Fract. 45-55, Table XII	0.85	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0112	10.46	19.5	6.31	6.88
10. No enzymatic digestion	Fract. 45-55, Table VII	0.15	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0112	10.46	18.9	6.48	7.18
11. No enzymatic digestion	Fract. 5, Table II	0.28	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0112	10.46	18.5	6.27	6.84

TABLE I—*Concluded*

Sample	Northrop corresponding sample	Concentration protein	Medium	pH	$\delta_{20}$	$\eta_{20}$	$t$	$s_t \times 10^{13}$	$s_b \times 10^{13}$
		per cent				milli-poise	°C.		
12. Normal plasma globulin fraction	Fract. 45-55, Table XII	0.9	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0112	10.46	19.0 <sub>6</sub>	6.20	6.86
13. Normal plasma globulin fraction	Fract. 45-55, Table XII	0.9	Phosphate 0.05 M plus 1 per cent $(\text{NH}_4)_2\text{SO}_4$	7.3	1.0112	10.76	18.6 <sub>6</sub>	6.25	6.98
14. No enzyme toxin-antitoxin complex	Table IX	0.24	Acetic acid 0.05 M	3.6	1.0025	10.15	19.7	$\begin{cases} 5.98 \\ 8.40 \end{cases}$	$\begin{cases} 6.26 \\ 8.80 \end{cases}$

sisted of several proteins of the same molecular size and shape but which reacted with different amounts of toxin.

Experiments performed with antitoxin preparations obtained without trypsin treatment are summarized in the second half of Table I. All preparations, whatever their titer in antitoxin, have the same sedimentation constant,  $s_{20}^{\text{water}} \approx 6.8 \times 10^{-13}$ . For example, Experiment 12 was carried out with a fraction obtained from normal plasma, the material of Experiment 8 contained only 15 per cent antibodies, whereas the ratio  $L_f/\text{PN}$ , of the material of Experiment 11, was as high as that found for the purest sample obtained by trypsin digestion (900  $L_f/\text{PN}$ ). However, the sedimentation constant of the three specimens was the same within experimental error. Some of the sedimentation patterns can be seen in Fig. 1.

It is worth mentioning that Kekwick and Record (6) recently came to the conclusion that there were at least two distinct diphtheria antitoxins, one present in the  $\beta$ -globulin and the other in the  $\gamma$ -globulin fraction of horse serum. They reported that the constant of sedimentation of the  $\beta$ -fraction was  $7.18 \times 10^{-13}$ , whereas that of the  $\gamma$ -fraction was  $6.87 \times 10^{-13}$  in agreement with the value reported here. Experiment 14 was made with the toxin-antitoxin complex dissolved in 0.05 M acetic acid. Two components could be detected in roughly the same amount, but the sedimentation constants corresponded neither to toxin nor antitoxin.

The diffusion constant was determined optically as previously described

(7). The measurements were made at  $20^\circ$  with the same sample, and the same medium used for Experiments 3 and 4 of Table I. The concentration in protein was 0.3 per cent. The diffusion constant was calculated from  $D = \frac{S^2}{4\pi t H_{\max}^2}$ , where  $S$  is the diffusion area in  $\text{cm}^2$ ,  $t$  the time in seconds, and  $H_{\max}$  the maximum height of the curve in centimeters. It was found, for this

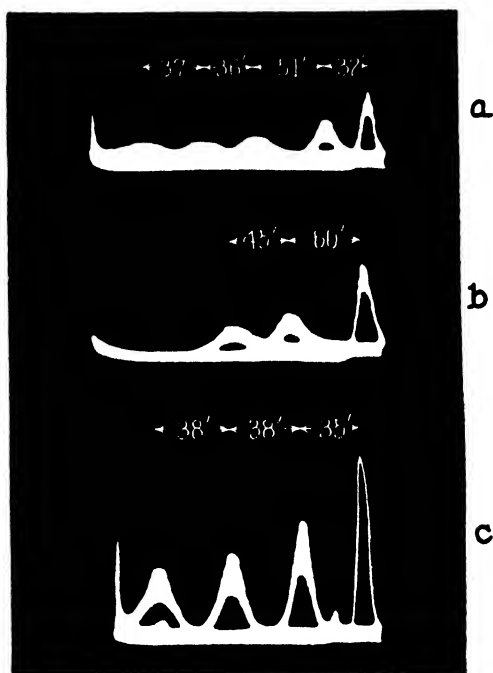


FIG. 1. All patterns obtained during the course of each sedimentation experiment have been superimposed on the same plate. *a*, *b*, and *c* refer to Experiments 2, 3 and 12 respectively (see Table I). A trace of the heavy component  $s_{20}^{\text{water}} = 18.0 \times 10^{-13}$  can be seen in Fig. 1 *c*.

particular medium,  $D_{20} = 5.56 \times 10^{-7}$  from which the calculated value  $D_{20}^{\text{water}} = 5.76 \times 10^{-7}$ .

Data have been summarized in Fig. 2 where the values  $\frac{1}{H^2}$  are plotted against  $t$ . As required by the theory, a straight line is obtained which extrapolated passes through the origin, which shows that no disturbance occurred at the time of formation of the boundary. This had been observed by Longworth (8) who used the linear relationship  $H$  against  $1/\sqrt{t}$  to represent his results on the diffusion of egg albumin. As seen from the diagram, seven "diffusion

areas" were determined; the relative values of the areas have been indicated inside the patterns, the constancy of the values is satisfactory. Since the ordinates of the diagram represent the  $\frac{1}{H^2}$  values, the relative heights  $H$  have been indicated for each pattern.

*Molecular Weight of the Purified Trypsin-Treated Antitoxin*

The molecular weight was calculated from the usual formula  $M = \frac{RTs}{\bar{D}(1 - V\rho)}$  with the numerical values,  $s_{20}^{\text{buffer}} = 5.02 \times 10^{-13}$  (Experiments 3 and 4),  $D_{20}^{\text{buffer}} = 5.56 \times 10^{-7}$ ,  $V_{20} = 0.749$ ,  $\rho_{20} = 1.0107$ , from which  $M = 90,500$ .

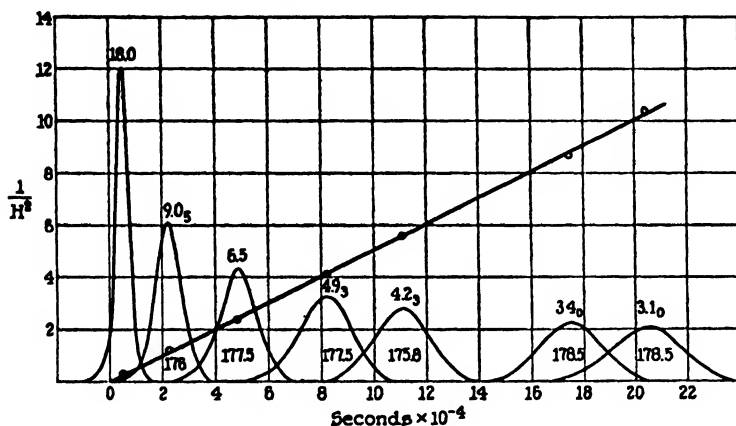


FIG. 2. Diffusion data.

The shape factor  $\frac{f}{f_0} = \frac{RT}{6\pi D\eta N} \frac{(4\pi N)^{\frac{1}{2}}}{(3VN)}$  was 1.23.

Petermann and Pappenheimer reported for their pepsin-treated sample  $D_{20}^{\text{water}} = 5.8 \times 10^{-7}$ ,  $\frac{f}{f_0} = 1.22$ , and  $M = 98,000$ .

It can then be concluded that the antitoxin molecule obtained by pepsin treatment has the same size and shape as the pure antitoxin obtained by trypsin treatment. The statement of Petermann and Pappenheimer that, "the increase in immunological potency of the digested antitoxin is directly proportional to its decrease in size," does not seem to hold in the case of the trypsin-treated antitoxin since, as mentioned above, the fraction used in Experiment 11 with a sedimentation constant of  $6.84 \times 10^{-13}$  was as active as the pure fraction of Experiment 6 with a sedimentation constant of  $5.65 \times 10^{-13}$ .

*Electrophoresis Experiments.*—Some of the results obtained can be seen in Fig. 3. Fig. 3 a represents the pattern of the descending boundary obtained,

205 minutes after the beginning of electrolysis, with the fraction used for centrifugation Experiment 7 (pH 2.9, specific conductivity 0.01095 at 0°). One component was present with a mobility  $\mu = 4.05 \times 10^{-5}$ . Current was reversed for 205 minutes and the pattern appeared as seen in Fig. 3 *a'*. There was no appreciable reversible spreading. Figs. 3*b*, 3*c*, 3*d*, correspond to fractions respectively 6, 8, and 12 of Table V of Northrop's article (1). The samples were dialyzed in the same phosphate buffer pH 7.3, 0.05 M (specific conductivity 0.00336 at 0°). Pictures *b*, *c*, and *d* were taken after electrolyzing for 3 hours, 3 hours, and 2½ hours respectively (8 volts/cm.). After reversal

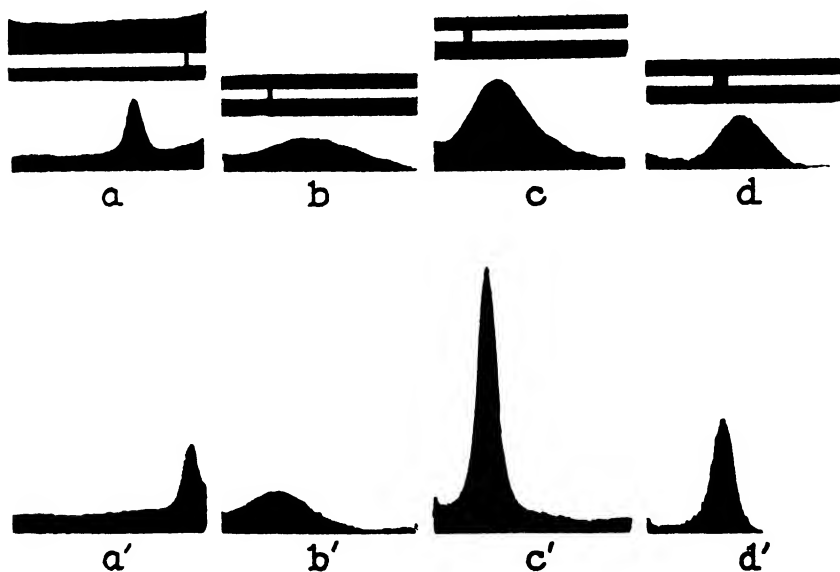


FIG. 3. Electrophoretic patterns of purified trypsin-treated antitoxin.

of the current electrolysis was continued for the same respective times and the appearance of the patterns was as shown in Figs. 3*b'*, 3*c'*, 3*d'*. Only one component could be detected moving very slowly towards the anode with a mobility  $\mu = -0.4 \times 10^{-5}$ . There was, however, a considerable amount of reversible spreading in all three samples, especially in No. 6. It has been often suggested that reversible spreading observed during electrophoresis of a protein near its isoelectric point is a measure of its electrical homogeneity. Since, in this case, the amount of reversible spreading was the same in two samples, one of them with constant solubility properties, the question arose whether another cause than heterogeneity could account for the reversible spreading. As suggested by Northrop (1), the phenomenon might be connected with electroendosmosis. Experiments were then made with samples

of fraction 8, one being dialyzed in veronal buffer  $M/15$ , pH 7.2 (specific conductivity 0.00273 at  $0^\circ$ ), the other in veronal buffer  $M/15$  plus  $M/20$   $\text{CaCl}_2$ , pH 7.1 (specific conductivity 0.00841 at  $0^\circ$ ). Both electrophoresis experiments were carried out with a current density of 0.027 amp./cm.<sup>2</sup> and the periods were so chosen as to make the products  $e \times t$  the same in both cases. Results

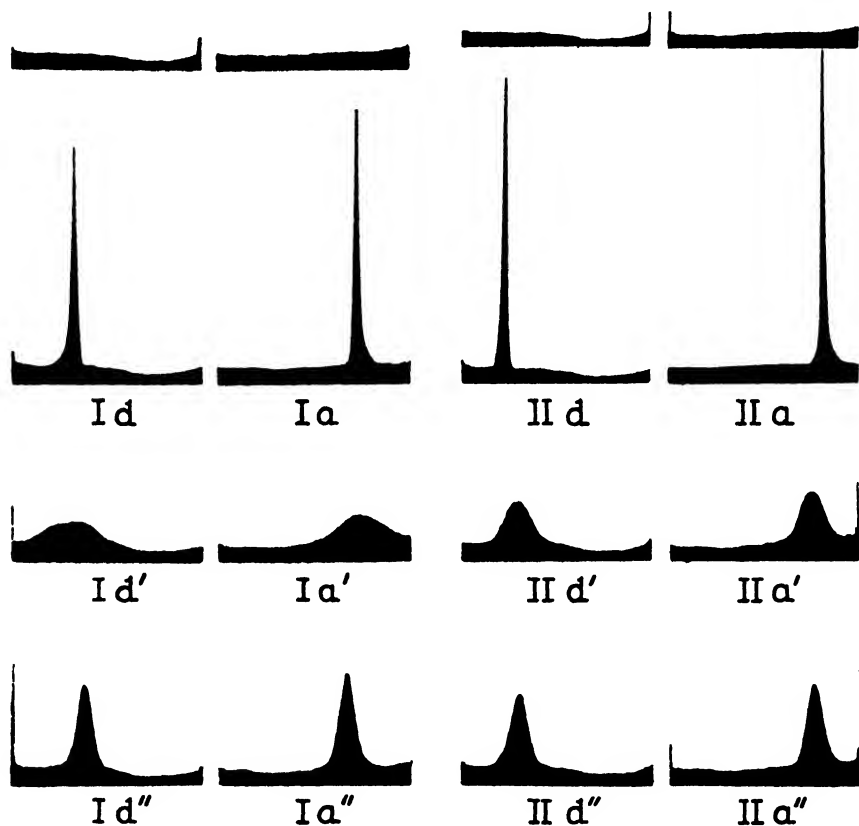


FIG. 4. Electrophoretic patterns of purified trypsin-treated antitoxin, demonstrating the effect of salts on reversible spreading.

can be seen in Fig. 4. I refers to veronal buffer, II refers to veronal plus  $\text{CaCl}_2$  buffer, "a" and "d" stand for ascending and descending boundaries, Ia and Id show the boundaries before electrolysis, Ia' and Id' after 85 minutes of electrolysis, and Ia'' and Id'' 87 minutes after reversal of current. The amount of reversible spreading is considerable and the difference in the pattern of the rising and descending boundary is small. IIa and IId are the initial boundaries, IIa' and IId' are the boundaries 240 minutes later, IIa'' and IId'', the boundaries 240 minutes after reversal of the current. The amount of

reversible spreading is small. The presence of  $\text{CaCl}_2$  has diminished the spreading.

It can be concluded that reversible spreading in this case is not a measure of the homogeneity of the material.

Some electrophoresis experiments were conducted to find out how different the pattern of an immune plasma would be from that of a normal plasma to which some purified trypsin-treated antibodies had been added. Dilute samples of normal plasma, immune plasma, and normal plasma to which was added the same number of units present in the immune plasma, were dialyzed against the same phosphate buffer, pH 7.3, 0.04  $M$  (specific conductivity 0.00356

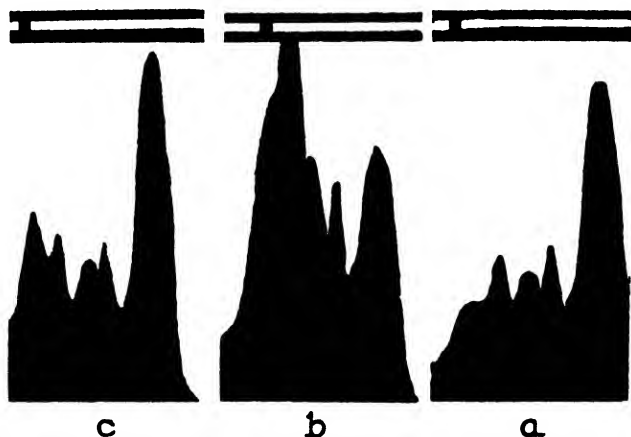


FIG. 5. Electrophoretic patterns of normal plasma, immune plasma, and normal plasma plus purified antibodies.

at  $0^\circ$ ). After dialysis, the samples of immune plasma and normal plasma plus antitoxin, contained about 250 units per milliliter. Electrolysis of each of the three samples was carried out with a current density of 0.027 amp./cm.<sup>2</sup> and the appearance of the pattern after  $1\frac{1}{2}$  hours can be seen in Fig. 5; *a* corresponds to the normal plasma, *b* to the immune, and *c* to the normal plus purified antitoxin (all descending boundaries). The differences are sharply marked. The addition of purified antitoxin has slightly enhanced the amount of the  $\gamma$ -globulin component of the normal plasma, whereas the pattern of the immune plasma is very different. There is a slight decrease of albumin, as so often noticed with immune plasma, a small increase of  $\alpha$ - and  $\beta$ -globulins, and a very large one of  $\gamma$ -globulin, so large, in fact, that it prevented the resolution of the fibrinogen component. The important conclusion to draw is that the large amount of  $\gamma$ -globulin present in the immune plasma contains either antibodies of much lower activity besides the pure antitoxin isolated, or a large amount of inert protein.

Measurement of the area under the different maxima of the patterns permits estimating the concentration of the corresponding components. As can be seen from Table II, the concentration of the  $\gamma$ -globulin fraction plus antitoxin in the immune plasma is 1.6% per cent as compared to 0.6 per cent for the corresponding fraction of normal plasma, plus purified antitoxin, having the same total immunological potency.

It appears from Table II that the large  $\gamma$ -fraction of the immune plasma has an average mobility nearly twice that of the  $\gamma$ -fraction of the normal plasma plus antitoxin. Since the fibrinogen component was not resolved in the pattern of the immune plasma, definite conclusions cannot be made. It is worth mentioning that Tiselius and Dahl (5) found that their immune glob-

TABLE II  
*Electrophoresis of Dilute Whole Plasma*

Protein constituent	Normal plasma		Immune plasma (250 units)/ml.		Normal plasma plus purified antibodies (250 units)/ml.	
	Concen- tration	Mobility	Concen- tration	Mobility	Concen- tration	Mobility
	per cent	$-u \times 10^5$	per cent	$-u \times 10^5$	per cent	$-u \times 10^5$
Total.....	3.5	—	4.8 <sub>5</sub>	—	3.65	—
Albumin.....	1.7 <sub>5</sub>	4.9	1.4	4.4	1.65	5.2
$\alpha$ -Globulin.....	0.40	3.2	0.53	2.9	0.40	3.3
$\beta$ -Globulin.....	0.39	2.5	0.78	1.8	0.40	2.6
Fibrinogen.....	0.48	1.5	0.4 <sub>5</sub>		0.42	1.4
$\gamma$ -Globulin, antibodies.....	0.43	0.4	1.6 <sub>5</sub>	0.9	0.64	0.4

ulin fraction with a mobility of  $2.0$  to  $2.4 \times 10^{-5}$  was gradually transformed, by treatment with pepsin, into a component merging into the  $\gamma$ -fraction.

*Ultracentrifuge.*—The apparatus previously described was used. The control of the temperature of the rotor has since been greatly improved. Following a suggestion of Dr. MacInnes of these laboratories, the possibility of making sedimentation experiments at low temperature was investigated. A cooling coil connected with a commercial frigidaire unit was installed inside the vacuum chamber. The chamber was insulated by two concentric cylinders of polished chrome-plated copper sheets which were placed between the cooling coil and the wall. Both steel end-plates of the vacuum chamber were insulated by discs, also made of chrome-plated sheets. Temperature of the rotor was measured by bringing the junction of an iron-constantan couple into contact with the rotor. This could be accomplished while the chamber was evacuated because the thermoelement was mounted on a rod which could move vertically through an air-tight bearing. With this arrangement the rotor could be kept at any temperature between  $-5^\circ$  and room temperature. In order to facilitate



heat exchange, a pressure of 0.15 mm. of hydrogen was maintained in the chamber for high speed centrifugation. All experiments were made at 57,600 R.P.M. Under these conditions the temperature of the rotor could be kept constant or nearly constant for hours if the temperature of the chamber was about  $10^\circ$  lower than that of the rotor. For instance, the temperature of the rotor during Experiment 12 (Table I) was  $18.8^\circ$  before centrifugation and  $19.3^\circ$  after a 2 hour run at 57,600 R.P.M., an increase of only  $0.25^\circ$  per hour, five times less than under a vacuum of  $10^{-5}$  mm. of Hg.

The rate of sedimentation was determined by the Philpot-Svensson "Schlieren" method. In a few cases measurements were duplicated with the Lamm "scale" method. Both methods gave the same value for the calculated constant of sedimentation within  $\pm 0.2$  per cent.

#### SUMMARY

Ultracentrifugation studies of diphtheria antitoxin showed that:

1. Purified antitoxin of high activity obtained from horse plasma without enzymatic treatment has exactly the same sedimentation constant as the globulin fraction obtained in a similar way from normal horse plasma  $s_{20}^{\text{water}} = 6.9 \times 10^{-13}$ .

2. Purified antitoxin obtained with trypsin digestion of the toxin-antitoxin complex has a sedimentation constant of  $s_{20}^{\text{water}} = 5.5 \pm 0.1 \times 10^{-13}$ , a diffusion constant of  $D_{20}^{\text{water}} = 5.7 \times 10^{-7}$ , and a molecular weight of about 90,000.

Electrophoresis experiments demonstrated that:

1. The trypsin-purified antitoxin has an isoelectric point not far from pH 7.0.
2. The reversible spreading noticed at about pH 7.3 cannot be attributed to heterogeneous preparation.
3. The large increase in the  $\gamma$ -globulin fraction occurring during immunization consists either of antitoxin of various degrees of activity or of some inert protein in addition to the antitoxin.

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# THE REVERSIBLE INACTIVATION OF TOBACCO MOSAIC VIRUS BY CRYSTALLINE RIBONUCLEASE

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The inactivation of plant viruses by enzymic action has been reported by several investigators (1-5). The inactivation may be irreversible as in the case of pepsin where virus protein is hydrolyzed (3) or reversible as in the case of trypsin where the inactivation appears to be due in part to a decreased susceptibility of the test plant in the presence of the enzyme (4). Besides its protein component tobacco mosaic virus also contains a ribonucleic acid, which thus far has proved necessary for virus activity. In experiments with phosphatase Pfankuch and Kausche (5) have demonstrated inactivation of tobacco mosaic and latent mosaic viruses and, although chemical proof was lacking, suggested that the loss of activity may be due to a splitting off of a phosphate group from the virus nucleic acid. It is unlikely that phosphatase would affect the nucleic acid present in a virus particle because it has no demonstrable effect on the free nucleic acid itself (6). The crystalline ribonuclease recently isolated by Kunitz, on the contrary, splits yeast ribonucleic acid into particles which have a higher diffusion rate than free ribonucleic acid and are not precipitated by glacial acetic acid (7). It also produces a similar change in the free virus nucleic acid and depending on the type of linkage between protein and nucleic acid in the virus might be expected to split off a portion of the nucleic acid. Preliminary experiments showed that the virus was inactivated by relatively low concentrations of ribonuclease. Studies on the rate of inactivation, on the effect of different enzyme concentrations and of dilution of the inactive virus-enzyme mixtures on virus activity suggested that inactivation was brought about by a combination of virus with ribonuclease. In more concentrated solutions and in the absence of salt, the virus-enzyme complex separated in the form of long fiber-like particles, which on analysis proved to contain about 14 per cent ribonuclease. That this inactive complex could be readily dissociated to give back virus was shown by recovery of about 100 per cent of the original virus activity from an inactive virus-enzyme complex and by the solubility behavior of the complex at different hydrogen ion concentrations. This paper presents the results of these experiments.

*Effect of Ribonuclease Concentration on Virus Activity.*—Preliminary experiments showed that the virus activity of solutions containing from  $10^{-3}$  to  $10^{-5}$  gm. of purified tobacco mosaic virus per ml. was strikingly reduced by the presence of relatively small amounts of crystalline ribonuclease.<sup>1</sup> In order to determine the effect quantitatively solutions were made up containing  $10^{-3}$  and  $10^{-5}$  gm. of virus per ml. respectively and enzyme concentrations in steps of ten from  $1.2 \times 10^{-9}$  gm. per ml. to  $1.2 \times 10^{-4}$  gm. per ml. in 0.1

TABLE I

*Effect of Ribonuclease Concentration on Virus Activity When Tested Immediately after Mixing*

Concentration (gm. protein per ml.)		<i>Phaseolus vulgaris</i> *				<i>Nicotiana glutinosa</i> *			
Virus	Ribonuclease	No. of lesions		Concentration of control (gm. per ml.)	Per cent of original activity	No. of lesions		Concentration of control (gm. per ml.)	Per cent of original activity
		Mixture	Control			Mixture	Control		
$10^{-5}$	$1.2 \times 10^{-6}$	4	4	$10^{-7}$	1.0	40	3	$10^{-7}$	10
	$1.2 \times 10^{-7}$	39	15	$10^{-7}$	2.6	149	3	$10^{-7}$	50
	$1.2 \times 10^{-8}$	135	70	$10^{-6}$	20	138	36	$10^{-6}$	40
	$1.2 \times 10^{-9}$	551	394	$10^{-5}$	140	251	203	$10^{-5}$	125
$10^{-3}$	$1.2 \times 10^{-4}$	6	6084	$10^{-3}$	0.1	36	312	$10^{-3}$	10
	$1.2 \times 10^{-5}$	14	6084	$10^{-3}$	0.2	133	312	$10^{-3}$	40
	$1.2 \times 10^{-6}$	317	6084	$10^{-3}$	5	424	312	$10^{-3}$	140
	$1.2 \times 10^{-7}$	1489	6084	$10^{-3}$	25	387	312	$10^{-3}$	130

\* With a concentration of  $10^{-5}$  gm. virus per ml., 10 to 16 half leaves were used for each mixture and its respective control: with a concentration of  $10^{-3}$  gm. virus per ml., 5 to 8 whole leaves were used both for the test solution and the controls.

† Calculated from the number of lesions obtained from the mixture and the control and

their respective virus concentrations. Per cent original activity =  $\frac{\text{No. of lesions (mixture)}}{\text{No. of lesions (control)}} \times$

$\frac{\text{Concentration of virus (control)}}{\text{Concentration of virus (mixture)}} \times 100.$

m phosphate buffer at pH 7. The activities of these solutions were then compared immediately by the half-leaf method (8) on leaves of *Nicotiana glutinosa* L. and *Phaseolus vulgaris* L. var. Early Golden Cluster with concentrations of virus which gave approximately the same number of lesions. The activities of the solutions were then calculated as percentage activity of the original virus solutions in each case. These data which are summarized in Table I show that an enzyme concentration of  $1.2 \times 10^{-9}$  gm. per ml. has little or no effect on virus activity at a virus concentration of  $10^{-5}$  gm. per

<sup>1</sup> The writer is indebted to Dr. M. Kunitz for the preparation of crystalline ribonuclease.

ml. In this more dilute virus solution a decrease in activity was observed with a concentration of  $1.2 \times 10^{-8}$  gm. ribonuclease per ml. when tested on Early Golden Cluster plants but the effect was not pronounced until a concentration of  $1.2 \times 10^{-7}$  or  $1.2 \times 10^{-6}$  gm. per ml. was reached. Some difference was observed in the behavior of the two types of test plants. As in earlier experiments with trypsin (4) the *P. vulgaris* plants were relatively more sensitive to the enzyme than *N. glutinosa* plants. With a virus concentration of  $10^{-3}$  gm. per ml., higher enzyme concentrations were required to show inactivation, indicating in the case of the more dilute virus solutions that the inactivation was not due entirely to a decreased susceptibility of the

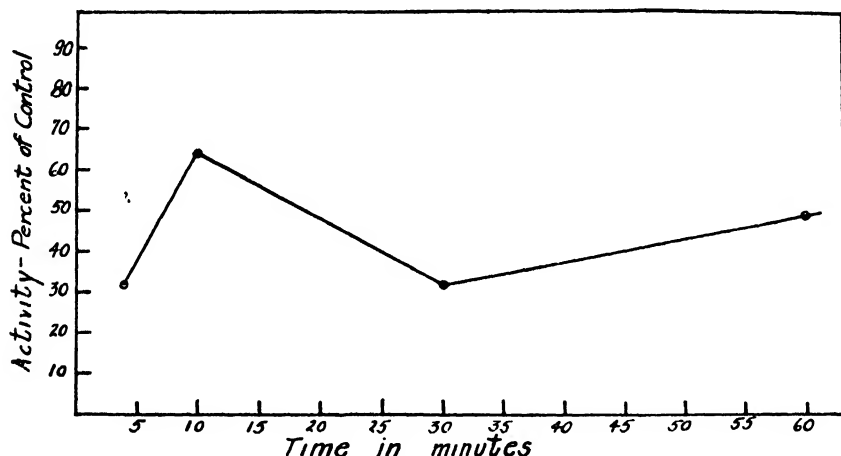


FIG. 1. Effect of time of standing on inactivation of tobacco mosaic virus by ribonuclease.

test plants in the presence of ribonuclease. In this connection it is of interest to compare the enzyme concentrations which produced inactivation with the concentrations of trypsin and those of various inert proteins which cause an inhibition of virus activity as studied by Stanley (4) and Ross and Stanley (9), respectively. The amount of ribonuclease required to cause almost 100 per cent inactivation is of the order of one-one hundredth the amount of trypsin and is about one-one hundred thousandth the amount of inactive proteins studied by Ross and Stanley (9), which produced about a 50 per cent inactivation.

*Rate of Inactivation of Virus.*—The question of the effect of time on the inactivation of virus was studied by comparing the activity of virus-enzyme mixtures with suitable controls immediately and after they had stood at room temperatures for varying periods of time up to 10 days. The results of a typical experiment are shown in Fig. 1. The mixture consisted of virus

at  $10^{-8}$  gm. per ml. and enzyme at  $1.2 \times 10^{-7}$  gm. per ml. in 0.1 M phosphate buffer at pH 7. The control was a solution containing  $10^{-8}$  gm. virus per ml. The control virus solution and the enzyme-virus mixture in each test were inoculated on 12 half leaves of *Phaseolus vulgaris* var. Early Golden Cluster. In all cases the activity after standing was not significantly different from that of the solution when tested immediately after mixing. There was no suggestion, therefore, that the inactivation was due to a progressive hydrolysis of virus.

TABLE II  
*Effect of Dilution on Activity of Virus-Ribonuclease Mixtures*

Virus-enzyme mixture (gm. protein per ml.)	<i>Phaseolus vulgaris</i> *				
	Dilution	No. of lesions		Concentration of control (gm. per ml.)	Per cent of original activity
		Mixture	Control		
Virus $10^{-8}$ Enzyme $1.2 \times 10^{-7}$	Undiluted	948	496	$10^{-8}$	1.9
	1-5	987	660	$10^{-8}$	7.5
	1-10	993	430	$10^{-8}$	23
	1-50	370	530	$10^{-8}$	35
	1-100	645	897	$10^{-8}$	70
	1-500	212	180	$2 \times 10^{-8}$	120
	1-1000	47	51	$10^{-8}$	97
Virus $10^{-8}$ Enzyme $1.2 \times 10^{-6}$	Undiluted	39	53	$10^{-8}$	0.07
	1-5	128	33	$10^{-8}$	2.0
	1-10	186	40	$10^{-8}$	4.6
	1-50	233	43	$10^{-8}$	27
	1-100	160	71	$10^{-8}$	22
	1-500	139	109	$10^{-8}$	64
	1-1000	120	87	$10^{-8}$	138

\* 12 to 16 half leaves were used for each mixture and its control solution.

† Calculated as in Table I.

*Effect of Dilution and the Reversal of Inactivation.*—It is well known that the dilution of inactive antigen-antibody complexes in many cases causes a partial reactivation of the antigen. If the observed inactivation of tobacco mosaic were due to an unspecific combination of virus with ribonuclease or to an inhibitory or toxic effect on the plant, then reactivation of inactive mixtures might also take place with dilution. The activities of several virus-enzyme mixtures were therefore compared at various dilutions with control solutions containing the same amounts of virus activity in the absence of enzyme. These results are summarized in Table II. It may be seen that a mixture of enzyme and virus which contained only a fraction of 1 per cent of the original virus activity was fully active when the solution was diluted from 1 to 500 to 1 to 1000 times.

It seemed apparent from these experiments that if a virus-enzyme complex were formed, it was readily dissociated. Attempts were made to restore the original specific activity of an inactive virus-enzyme mixture by repeated differential ultracentrifugation. Free or combined virus would be expected to sediment in a centrifugal field which would leave the uncombined enzyme in the supernatant liquid. Re-solution of the pellet in dilute phosphate buffer and sedimentation would leave an additional portion of enzyme in the supernatant liquid and repetition of the process should finally result in the elimination of all the enzyme.

TABLE III

*Recovery of Virus Activity from an Inactive Virus-Enzyme Mixture by Repeated High Speed Centrifugation*

Treatment	No. of lesions on <i>Phaseolus vulgaris</i> and activity					
	Experiment 1			Experiment 2		
	Mixture	Control	Per cent of* original activity	Mixture	Control	Per cent of* original activity
(1) $10^{-3}$ gm. virus + $2.7 \times 10^{-4}$ gm. ribonuclease per ml.	0	2343	0			
(2) Above mixture centrifuged and sediment suspended in 0.1 M phosphate	127	756	17	273	1020	27
(3) Solution of sedimented virus from (2) treated as in (2)	1193	1761	68	635	770	82
(4) Solution of sedimented virus from (3) treated as in (2)	487	670	73	476	676	70
(5) Solution of sedimented virus from (4) treated as in (2)	198	325	61	680	741	92

\*No. of lesions (mixture)  
No. of lesions (control)  $\times 100$ .

*Experimental Procedure.*—0.21 ml. of ribonuclease solution containing  $2.7 \times 10^{-4}$  gm. ribonuclease per ml. was added to 14 ml. of purified tobacco mosaic virus containing 1 mg. of virus per ml. in 0.1 M phosphate buffer. This solution and the control containing an equal amount of virus were centrifuged in stainless steel tubes at 550 R.P.M. for  $1\frac{1}{2}$  hours and the supernatant liquids were poured off. 10 ml. of 0.1 M phosphate at pH 7 were added to each tube and the virus pellets were redissolved. 0.1 ml. samples were removed from each tube, diluted with 9.9 ml. of 0.1 M phosphate at pH 7, and inoculated by the half-leaf method on 12 to 16 leaves of *Phaseolus vulgaris* var. Early Golden Cluster. The remaining solution was subjected to three additional centrifugal cycles, samples being removed as described above after each sedimentation.

After one sedimentation the specific activity of the sedimented material as shown in Table III was from 17 to 27 per cent that of untreated virus.

After two sedimentations the activity was about 70 per cent and after four sedimentations the activity of the treated virus was about the same as that of the original sample.

*Crystallization and Analysis of Virus-Enzyme Complex.*—When an enzyme solution was added to a solution of virus containing  $10^{-3}$  gm. per ml. or more, in 0.1 *M* phosphate buffer at pH 7, it was observed that a precipitate formed which redissolved when the solution was mixed. When a dialyzed enzyme solution was added to a virus solution in the absence of salt, however, the precipitate which separated failed to redissolve. Microscopic examination

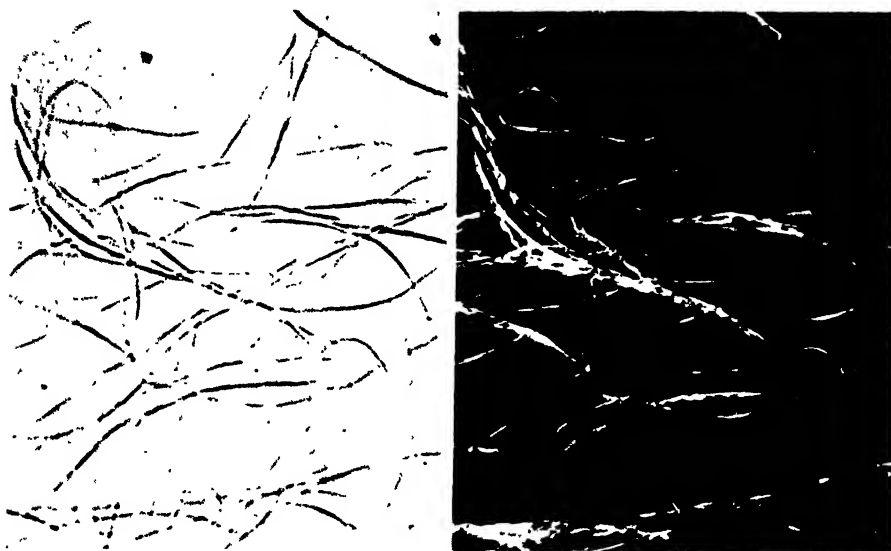


FIG. 2. Fiber crystals of tobacco mosaic virus-ribonuclease complex. 240  $\times$ . Photographed in light and dark fields respectively.

showed the precipitate to consist of needles similar to those obtained when the purified virus is treated with ammonium sulfate. The careful addition of enzyme to a stirred-virus solution in the absence of salt resulted in the separation of long fibers as shown in Fig. 2. Nitrogen analyses of the supernatant liquids obtained after successive additions of enzyme showed that the amount of soluble nitrogen decreased to a minimum which was almost zero and then as more enzyme was added the value increased. The precipitate was insoluble in distilled water even after four washings in the centrifuge but could be dissolved readily by the addition of a trace of acid or alkali or phosphate buffer at pH 7.

In order to determine whether or not the precipitate consisted of virus alone or of virus in combination with enzyme or whether phosphorus had

been split off, nitrogen and phosphorus determinations were run on the washed precipitate and on the supernatant liquid and washings from a known quantity of virus and enzyme.

*Experimental Procedure.*—In Experiment 1, 0.2 ml. of an aqueous ribonuclease solution containing 2.84 mg. of ribonuclease nitrogen per ml. was added slowly with stirring to 10 ml. of tobacco mosaic virus solution containing 2.09 mg. of virus nitrogen. The resulting precipitate was centrifuged at 2200 R.P.M., the supernatant liquid was carefully removed with a dropping pipette, and the precipitate was washed four times in the centrifuge with 2 ml. portions of distilled water. The first supernatant liquid and the washings were combined, and the insoluble precipitate resuspended in water was dissolved by the addition of a drop of 0.1 N NaOH. Aliquots of both solutions were then taken for nitrogen and phosphorus analyses. In Experiment 2,

TABLE IV

*Distribution of Nitrogen and Phosphorus in Precipitate and Supernatant Liquid after Mixing Known Quantities of Virus and Ribonuclease*

2.09 mg. of virus nitrogen + 0.568 mg. ribonuclease nitrogen

Experiment	Insoluble precipitate		Supernatant liquid and washings		Recovery	
	Nitrogen*	Phosphorus*	Nitrogen	Phosphorus	Nitrogen	Phosphorus
	mg.	mg.	mg.	mg.	per cent	per cent
1	2.43	0.077	0.27	<0.001	102	103
2	2.40	0.074	0.26	<0.001	100	99

\* Nitrogen and phosphorus determined by the methods of Levy and Palmer (10) and King (11) respectively.

the same procedure was followed with the exception that the ribonuclease added was dissolved in 2 ml. of water.

The results of two experiments are shown in Table IV. Because all the phosphorus was present as virus and was recovered in the precipitate, it can be concluded that the virus was quantitatively precipitated and that no nucleic acid was split off. A comparison of the nitrogen to phosphorus ratio of the precipitate with that of the original virus showed some additional enzyme nitrogen was also precipitated and was present in sufficiently strong combination to remain with the virus even after the precipitate had been washed four times.

The fact that a relatively insoluble virus-enzyme complex can be prepared provides evidence that the loss of virus activity when enzyme is added to virus in the absence of salt is due at least in part to the formation of such a complex. A comparison of the relative isoelectric points of the virus, pH 3.5,



and the enzyme about pH 8, shows that in a neutral solution such a complex would, in fact, be expected.<sup>2</sup>

#### DISCUSSION

The fact that no nucleic acid fraction is split off when ribonuclease is added to the active virus provides some insight into the type of combination between the protein of the virus and its nucleic acid and the nature of ribonuclease activity. Nucleic acid would appear to occupy an integral rather than a terminal position in the virus particle, for in the latter case hydrolysis by ribonuclease might be expected. Other evidence for such a hypothesis is the fact that the liberation of nucleic acid by treatment with alkali or urea is accomplished only with the destruction of the large virus particle into much smaller protein and nucleic acid components, as shown by the low rates of sedimentation of the split products (12, 13). In so far as ribonuclease activity is concerned it is evident that in the case of the tobacco mosaic virus nucleoprotein, ribonuclease fails to hydrolyze the linkage between nucleic acid and protein.

The inactivation with ribonuclease appears to be comparable to that found for other proteins with high isoelectric points, and is probably related to the formation of complexes between oppositely charged protein particles. The complex between virus and ribonuclease appears to be of the same type as that reported by Bawden and Pirie (14) to result from the action of clupein and papain on tobacco mosaic virus and as the ovalbumin-nucleic acid complex described by MacInnes and Longworth (15). The complex formed with tobacco mosaic virus unlike the latter, however, is insoluble in distilled water and gives the characteristic crystals or fibers. The solubility in salt or slightly acid or alkaline solutions suggests either partial or complete dissociation under these conditions as is the case for the ovalbumin-nucleic acid complex.

#### SUMMARY

The reversible inactivation of tobacco mosaic virus by crystalline ribonuclease is reported. Studies on the effect of time of standing on the amount of inactivation, and on the effect of dilution and repeated high speed centrifugation on the recovery of virus activity, and the preparation of an insoluble virus-enzyme complex show that the inactivation is brought about at least in part by a combination between virus and enzyme. The significance of the fact that ribonuclease has no detectable effect on the virus nucleic acid when the latter is in combination with protein in the form of virus is discussed with respect to the structure of the virus.

<sup>2</sup> A similar insoluble complex was formed with the bushy stunt virus and ribonuclease but in this case the precipitate was not definitely crystalline.

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# AN ELECTROPHORETIC STUDY OF MIXTURES OF OVALBUMIN AND YEAST NUCLEIC ACID

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## INTRODUCTION

In an ideal electrophoresis of a solution of two components the volumes swept through by the rising and descending boundaries due to each component are identical and are proportional to the mobilities of the separate components. Moreover, the area under each "peak" in the electrophoretic pattern is proportional to the stoichiometric concentration of that component in the mixture to which it is due. In this ideal or limiting case the patterns for the two sides of the channel of the Tiselius electrophoresis cell are mirror images of each other.

The two patterns are, however, never exact mirror images. The authors have already discussed<sup>1</sup> the asymmetries, including the  $\delta$  and  $\epsilon$  effects, that are observed in the electrophoretic patterns of a single component and deviations of this type are, of course, to be expected in the electrolysis of mixtures. In the case, however, of some mixtures asymmetries were observed<sup>2</sup> which appeared to be due to interaction between the constituents. This led to the research, to be described below, in which the effect of interaction of components on electrophoretic patterns has been investigated further, using mixtures of ovalbumin and yeast nucleic acid. It is also the purpose of this paper to indicate a method for the electrophoretic analysis of mixtures in which certain types of interaction occur, and to discuss the manner in which the asymmetries in the electrophoretic patterns arise.

## EXPERIMENTAL

The ovalbumin used in this research was prepared by the method of La Rosa<sup>3</sup> and was recrystallized three times. The nucleic acid was a sample prepared under the direction of Dr. P. A. Levene of these Laboratories. This material was electrophoretically homogeneous, *i.e.* gave a single sharp peak,

<sup>1</sup> Longworth, L. G., and MacInnes, D. A., *J. Am. Chem. Soc.*, 1940, **62**, 705.

<sup>2</sup> Longworth, L. G., Cannan, R. K., and MacInnes, D. A., *J. Am. Chem. Soc.*, 1940, **62**, 2580.

<sup>3</sup> La Rosa, W., *Chemist-Analyst*, 1927, **16**, 3.

other than the  $\delta$  and  $\epsilon$  effects, in the electrophoretic pattern. It was observed, however, that some of the acid was lost on dialysis in cellophane tubing. Hence it was necessary to dialyze for a definite interval and correct for the material lost.

The patterns of Fig. 1 were obtained in the electrophoresis of a mixture of 1.15 per cent ovalbumin,  $P$ , and 0.50 per cent nucleic acid,  $N$ , in a 0.1  $N$  sodium acetate buffer at pH 5.34. In this buffer both components carry appreciable negative charges, the mobilities having the values  $u_P = -2.8 \times 10^{-5}$  and  $u_N = -13.1 \times 10^{-5}$ . Under these conditions a pattern for the mixture is essentially the sum of the patterns for the two components which were obtained separately.

The patterns of Fig. 2 were obtained with a similar mixture, 1.15 per cent  $P$  and 0.67 per cent  $N$ , but in a 0.1  $N$  sodium acetate buffer at pH 4.63. In this solvent the protein still has a small negative mobility,  $-0.2 \times 10^{-5}$ , but the patterns exhibit asymmetries<sup>4</sup> that cannot be explained in terms of the  $\delta$  and  $\epsilon$  effects alone. Thus the displacements of the boundaries  $b$  and  $\gamma$ , Fig. 2, are proportional to the normal mobilities  $u_P$  and  $u_N$ , respectively, but the displacement of the boundary  $\beta$  corresponds to a mobility some eight times greater than the mobility of ovalbumin at this pH, while the displacement of the boundary  $c$  corresponds to a mobility appreciably less than that of nucleic acid. Moreover, the area under the boundary at  $\gamma$  corresponds, after correction for the dilution due to the  $\delta$  effect,<sup>1</sup> to only 0.56 per cent of nucleic acid whereas the actual concentration of that component was 0.67 per cent. Also the area under the boundary at  $c$  would correspond to 0.93 per cent if this were due entirely to nucleic acid. The results of this experiment are, as will be shown below, consistent with the assumption that the boundaries  $\gamma$  and  $b$  are due to nucleic acid and ovalbumin, respectively, moving with their normal mobilities, but whose concentrations have been modified by interaction. The boundaries  $\beta$  and  $c$  will, on the other hand, be shown to arise from the complexes due to combination of the components. It will be shown that the displacement of these boundaries involves the equilibrium between the components and the complex in addition to the motion of these substances in the existing electric field.

Although the patterns are not reproduced in this paper, experiments have been performed in which each of the four variables, *i.e.* pH, ionic strength,

<sup>4</sup> We have observed similar asymmetries in the patterns of mixtures of ovalbumin and salmine, which is a basic protamine, except that in this case the patterns approach those of a normal mixture at pH values *below* the isoelectric point of the protein.

<sup>5</sup> It may be noted in Fig. 2 that the gradients of refractive index in the boundary at  $c$  are not symmetrical about the ordinate passing through the maximum gradient. In measuring the displacement of this boundary it is necessary, therefore, to follow the procedure suggested by one of us (Longworth, L. G., *Ann. N. Y. Acad. Sc.*, 1941, **41**, 267).

protein concentration, and nucleic acid concentration, have been altered systematically. The results, some of which are given in Table I, indicate that

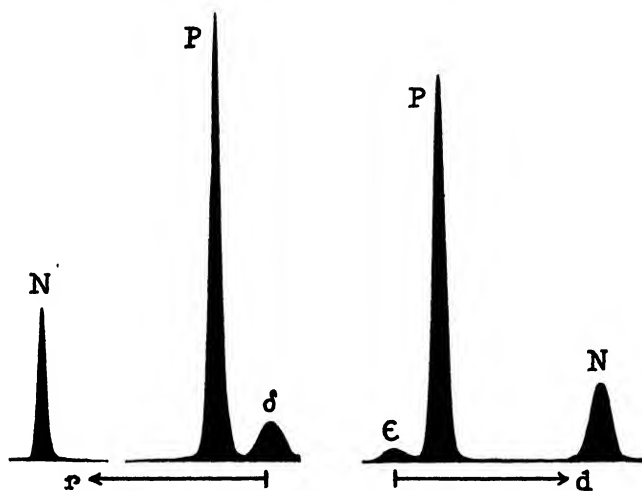


FIG. 1

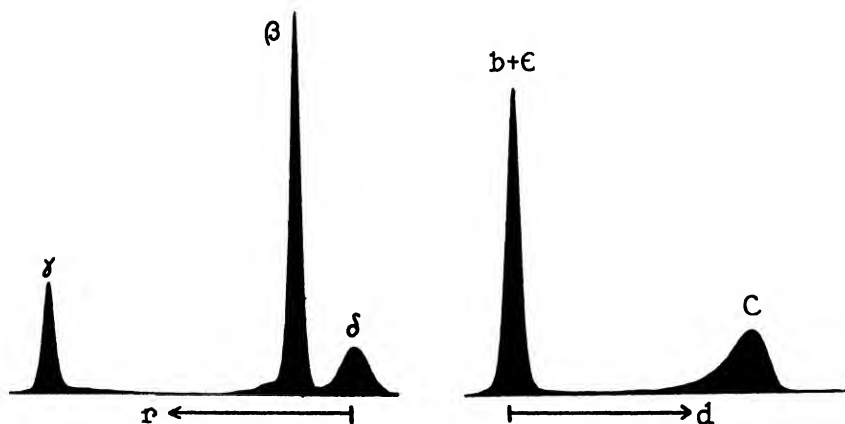


FIG. 2

FIG. 1. Electrophoretic patterns of a mixture of ovalbumin, 1.15 per cent, and yeast nucleic acid, 0.50 per cent, in a 0.1 N sodium acetate buffer at pH 5.34. The patterns were obtained after electrophoresis for 5000 seconds at 7.22 volts per cm.

FIG. 2. Electrophoretic patterns of a mixture of ovalbumin, 1.15 per cent, and yeast nucleic acid, 0.67 per cent, in a 0.1 N sodium acetate buffer at pH 4.63. The patterns were obtained after electrophoresis for 7000 seconds at 6.97 volts per cm.

pattern asymmetries of the type described above are enhanced by decreasing the ionic strength or by increasing the concentrations of the protein or the nucleic acid or both. The patterns of Figs. 1 and 2 illustrate the important

rôle that pH plays in such experiments. Experiments at pH values below 4.6, the isoelectric point of ovalbumin, are complicated by the partial precipitation of the components. For electrophoretic study, removal of the precipitate that forms on dialysis of the mixture<sup>6</sup> is not sufficient since additional precipitation frequently occurs, during an experiment, that interferes with observation of the boundaries. Thus in an experiment at pH 4.45, with conditions otherwise similar to those represented by Fig. 2, only a slight precipitate formed on dialysis and this was removed before electrolysis but during the latter procedure an optically opaque stratum of precipitate formed in the channel between the  $\delta$  and the  $\beta$  boundaries on the anode side. This precipitation was probably due to the decreased ionic strength in this region of the cell arising from the  $\delta$  effect and suggests that the solubility of the ovalbumin-nucleic acid complex is very sensitive to the ionic strength as well as to the pH.<sup>7</sup>

#### *Electrophoretic Analysis of Ovalbumin-Nucleic Acid Mixtures*

The distribution of the components in the cell before and after electrophoresis is shown in Figs. 3 and 4. The boundaries between the buffer solution and solution (in the buffer) of ovalbumin at an initial or total concentration  $p_i$  and nucleic acid at a total concentration  $n_i$  were present in the planes  $a$  and  $\alpha$  of Fig. 3 at the time the potential was applied. After passage of a current the boundaries in the anode side of the channel (Fig. 4) had swept through the volumes  $V_\gamma$  and  $V_\beta$ , while on the cathode sides the volumes were  $V_b$  and  $V_c$ .

The material recovered from the channel between the boundaries at  $\beta$  and  $\gamma$  was found by direct experiment to be pure nucleic acid at a "separated" concentration  $n_s$ , whereas that between the boundaries at  $b$  and  $c$  was pure ovalbumin at a corresponding separated concentration  $p_s$ . Since the passage of an electric current does not produce changes of composition in the body of a

<sup>6</sup> The precipitate formed in a 15 ml. sample of a 1.15 per cent  $P$ -0.58 per cent  $N$  mixture in a 0.1  $N$  sodium acetate buffer at pH 4.34 was separated, dissolved in 15 ml. of a 0.1  $\mu$  sodium phosphate buffer at pH 6.80, dialyzed against this buffer, and analyzed electrophoretically. The pattern indicated no interaction at this pH and corresponded to a mixture of 0.40 per cent  $P$  and 0.13 per cent  $N$ . Moreover the ovalbumin thus separated electrophoretically from the nucleic acid was still native and showed the same relative amounts of the  $A_1$  and  $A_2$  modifications (*cf.* footnote 2) as in the original stock solution of the protein. It thus appears that precipitation under the conditions outlined here does not denature the protein and that the complex with nucleic acid is reversibly dissociated at pH values sufficiently above the isoelectric point of the ovalbumin.

<sup>7</sup> Our observations concerning the influence of pH and ionic strength upon the solubility of the ovalbumin-yeast nucleic acid complex are in qualitative accord with those of Hammarsten and Hammarsten (Hammarsten, E., and Hammarsten, G., *Acta med. Scand.*, 1928, **68**, 199) on the complex formed by the related material, thymus nucleic acid, and ovalbumin.

homogeneous solution the protein solution in the bottom section of the channel, and the buffer solution in the two sides of the top section, remain unchanged. Moreover, ovalbumin and nucleic acid neither enter nor leave the cell.<sup>8</sup> Consequently if the pattern for one side of the channel indicates a loss by electrophoretic migration of one of these components the pattern for the other side should indicate a corresponding gain of that component.

Referring to Fig. 4, the quantity of protein initially present in the volume  $V_c$  was  $V_c p_t$  while that present in the same volume after electrolysis was  $(V_c - V_b)p_s$ . Thus the loss of this component from the cathode side was

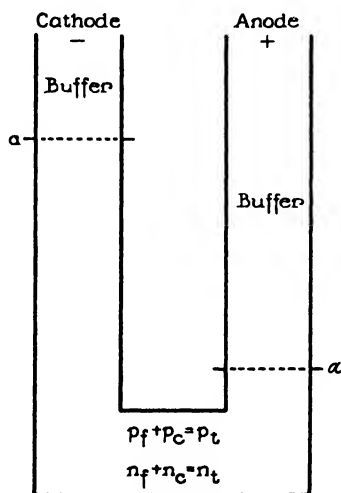


FIG. 3

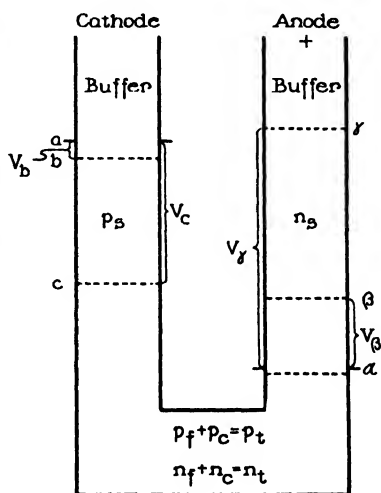


FIG. 4

FIG. 3. Diagrammatic representation of the initial distribution of materials in the electrophoresis channel.

FIG. 4. Distribution of materials in the channel after electrophoresis.

$V_c p_t - (V_c - V_b)p_s$  and this should equal the gain on the anode side, namely,  $V_\beta p_s \rho_P$  in which  $\rho_P$  is the dilution factor of the protein at the  $\delta$  boundary. Therefore,

$$V_c p_t - (V_c - V_b)p_s = V_\beta p_s \rho_P \quad (1)$$

and similarly for the nucleic acid,

$$V_c n_t = V_\beta n_t \rho_N + (V_\gamma - V_\beta)n_s \quad (2)$$

<sup>8</sup> The buffer electrolytes, on the other hand, move through the cell but the quantity of these materials present at any instant remains constant, except for negligibly small effects due to volume changes accompanying electrophoretic separation of the components if the partial volumes of the latter are not additive.



in which  $\rho_N$  is the corresponding dilution factor for the nucleic acid. All of the terms in equation 1, for example, can be obtained from the electrophoretic patterns of the rising and descending boundaries, with the exception of  $p_t$ , if the specific refractive increment of the protein is known. Thus from the two patterns the total concentration of protein,  $p_t$ , may be computed whether interaction takes place or not. This is obviously also true of equation 2, and  $n_t$ . Since the composition of the initial solution, and therefore  $p_t$  and  $n_t$ , were known a comparison of the observed and computed concentrations affords a test of our interpretation of the phenomena occurring during electrophoresis.

In making computations with the aid of equations 1 and 2 it will be assumed that the dilution factors are given by the relation

$$\rho_P = \rho_N = (A_t - A_\delta)/A_t \quad (3)$$

in which  $A_t$  is the total area of the electrophoretic pattern and  $A_\delta$  that due to the  $\delta$  boundary. The assumption that  $\rho_P = \rho_N$  is in accord with the theory of Henry and Brittain<sup>9</sup> who showed, for a somewhat simpler system, that at the  $\delta$  boundary "the advancing column will hold its constituent ions in the same relative proportion as in the original sol." The additional assumption contained in equation 3; namely, that  $\rho = (A_t - A_\delta)/A_t$ , represents an approximation based upon the observation that the gradients of buffer salts in the  $\delta$  boundary, being similar to those in the  $\epsilon$  boundary, are small in comparison with the gradients of  $P$  and  $N$ .

Equation 1 may be rearranged to give

$$p_t = \frac{V_c - V_b}{V_c - V_\beta \rho} p_s = \frac{V_c - V_b}{V_c - V_\beta \rho} k_P A_b \quad (1')$$

in which  $A_b$  is the area under the boundary at  $b$  and  $k_P$  is a factor converting this area into protein concentration. This factor, whose value is 0.003776, depends only upon constants of the apparatus and the specific refractive increment of ovalbumin, taken as 0.00184 for the Hg blue and violet lines used in the present research. Similarly, equation 2 may be rearranged to

$$n_t = \frac{V_\gamma - V_\beta}{V_c - V_\beta \rho} n_s = \frac{V_\gamma - V_\beta}{V_c - V_\beta \rho} k_N A_\gamma \quad (2')$$

in which  $k_N = 0.005185$ , the specific refractive increment for nucleic acid being taken as 0.0013.<sup>10</sup>

<sup>9</sup> Henry, D. C., and Brittain, J., *Tr. Faraday Soc.*, 1933, **29**, 798.

<sup>10</sup> This value is taken from the work of Seibert and Watson (Seibert, F. B., and Watson, D. W., *J. Biol. Chem.*, 1941, **140**, 55). It may be noted in this connection that the conclusions of our paper are actually independent of the values of the specific refractive increments since the concentrations of our stock solutions of both ovalbumin and nucleic acid were determined refractometrically.

The data necessary for computations with the aid of the foregoing relations, as obtained in five experiments in which the relative proportions of ovalbumin and nucleic acid were varied, are given in Table I. The data obtained from the patterns of Fig. 2 are given in the fourth column of this table. The other patterns were qualitatively similar. The areas (lines 1 to 5) under the peaks of these patterns are in arbitrary planimeter units whereas the displacement volumes (lines 6 to 9) are in milliliters per second per unit potential gradient. Line 10 of Table I contains the concentration of ovalbumin used in each experiment whereas line 11 contains the value of the concentration of albumin computed with the aid of equation 1'. Lines 12 and 13 contain the corre-

TABLE I

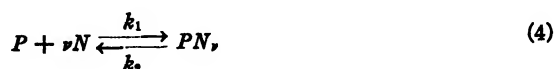
*Electrophoretic Analysis of Mixtures of Ovalbumin and Yeast Nucleic Acid in 0.1 N Sodium Acetate at pH 4.63 and 0°C.*

Experiment No.	1	2	3	4	5
1. $A_b$	133.0	273.0	254.5	255.5	231.0
2. $A_c$	149.5	84.5	151.5	179.5	296.0
3. $A_\beta$	146.0	294.0	274.5	265.5	247.5
4. $A_\gamma$	96.0	33.5	68.0	89.5	154.0
5. $A_\delta$	44.0	29.0	60.0	77.5	120.5
6. $V_b \times 10^5$	0.34	0.26	0.30	0.33	0.45
7. $V_c \times 10^5$	10.46	8.49	9.18	9.43	9.89
8. $V_\beta \times 10^5$	2.35	1.10	1.94	2.37	3.86
9. $V_\gamma \times 10^5$	13.02	12.36	12.76	12.94	13.47
10. Ovalbumin taken, per cent.	0.58	1.15	1.15	1.15	1.15
11. Ovalbumin computed, equation 1'.	0.60	1.13	1.13	1.17	1.19
12. Nucleic acid taken, per cent.	0.67	0.25	0.52	0.67	1.16
13. Nucleic acid computed, equation 2'.	0.63	0.26	0.51	0.66	1.11

sponding values for nucleic acid. The average difference between the observed and computed values is 2.4 per cent for the ovalbumin and 3.5 per cent for the nucleic acid. It is of interest that if interaction were neglected and the area  $A_\beta$  interpreted as due entirely to ovalbumin the average difference between the observed and computed values of  $p_i$  would be 6.9 per cent whereas if the area  $A_c$  were interpreted as due entirely to nucleic acid the average difference between the observed and computed values of  $n_i$  would be 45 per cent.

#### *The Interaction between Ovalbumin and Yeast Nucleic Acid*

In considering the probable nature of the interaction between ovalbumin and nucleic acid it will be assumed that they combine reversibly to form a complex  $X(=PN,)$



in which the velocity constant for the forward reaction is  $k_1$  and for the reverse reaction  $k_2$ , and the equilibrium constant is  $K = \frac{k_2}{k_1}$ . The available evidence indicates that the complex has a mobility,  $u_x$ , intermediate between  $u_p$  and  $u_N$ . For the purpose of this discussion no restriction is placed on  $\nu$ . It doubtless varies with both pH and ionic strength and may vary, as in the precipitin reaction,<sup>11</sup> with the concentrations of  $P$  and  $N$ . In the electrophoresis of such a mixture the equilibrium shown in equation 4 is not disturbed in the body of the solution by the migration of the constituents  $P$ ,  $N$ , and  $X$ . At the boundaries, however, the tendency of the components to separate, due to their mobility differences, is accompanied by a disturbance of the equilibrium. The extent to which the equilibrium will shift to compensate for the altered conditions produced by electrophoretic separation depends upon the magnitudes of the velocity constants in comparison with the rate of separation. The following cases may be distinguished.

1. If  $k_1$  is small and  $k_2$  large,  $K$  will also be large. Under these conditions the complex is essentially completely dissociated and the mixture will behave as a normal mixture of  $P$  and  $N$ .
2. If  $k_1$  is large and  $k_2$  small then  $K$  will be small. This system behaves like a mixture of the complex and either  $P$ <sup>12</sup> or  $N$ , depending upon which is in excess. If neither is in excess a single boundary, due to  $X$  alone, will be present in each side of the channel.
3. If  $k_1$  and  $k_2$  are both small and of similar magnitudes then  $K$  will be near unity. In this case finite concentrations of  $P$ ,  $N$ , and  $X$  will exist at equilibrium. The mixture will behave like a normal mixture of the three components since the adjustment of the equilibrium is slow in comparison with the rate of electrophoretic separation.
4. If the rate of adjustment of the equilibrium is comparable with the rate of

<sup>11</sup> See, for instance, Kendall, F. E., *Ann. N. Y. Acad. Sc.*, in press.

<sup>12</sup> From the description by Stenhagen and Teorell (Stenhagen, E., and Teorell, T., *Tr. Faraday Soc.*, 1939, **35**, 743) of their electrophoresis experiments on mixtures of serum albumin and thymus nucleic acid it appears possible that this system corresponds to case 2. Thus only two boundaries were present in each side of the channel and the patterns appeared to be symmetrical. Their analyses showed both nucleic acid and protein to be present in the region between the two boundaries on the anode side but essentially pure protein in the corresponding region on the cathode side, thus indicating protein to be present in excess.

Seibert (Seibert, F. B., *J. Biol. Chem.*, 1940, **133**, 593) has observed somewhat similar phenomena in her electrophoretic studies of the naturally occurring mixture of protein and nucleic acid from the tubercle bacillus. It is of considerable interest that her separation of these components, by salt precipitation, was much more effective at alkaline reactions, where both components are negatively charged, than at acid reactions.

electrophoretic separation, the behavior will be difficult to predict although one would expect the pattern to depend upon the rate of separation of the constituents.

5. If  $k_1$  and  $k_2$  are both large and of the same order of magnitude the equilibrium is adjusted as rapidly as required by the electrophoretic separation of the components. Consequently only two boundaries, aside from the  $\delta$  and  $\epsilon$  effects, appear in each side of the channel but the patterns are quite different from those of a normal mixture of two components.<sup>13</sup> The patterns shown in Fig. 2 and those for which data are given in Table I appear to be compatible with the conditions postulated in this last case. It is of interest to visualize how these patterns may arise.

The initial conditions in the electrophoresis cell may be represented by Fig. 3 in which the concentrations of combined, free, and total protein in the mixture are  $p_r$ ,  $p_f$ , and  $p_t$ , respectively, and the corresponding concentrations of nucleic acid are  $n_r$ ,  $n_f$ , and  $n_t$ . The conditions in the cell after electrophoresis are indicated diagrammatically in Fig. 4 in which, as has been stated, pure  $N$  is present at a concentration  $n_s$  in the region between the two leading boundaries on the anode side and pure  $P$  at a concentration  $p_s$  in the region between the two boundaries on the cathode side. The sequence of events leading from the initial to the final state is as follows.

In the anode side of the channel the free  $N$  in the mixture escapes and moves upward with its normal mobility to a position  $\gamma$ . The complex  $X$  also moves upward through the plane  $\alpha$  but in so doing tends to leave the slower moving  $P$  behind. Consequently a portion of the complex  $X$  dissociates in order to maintain the equilibrium indicated in equation 4. The  $N$  resulting from this dissociation moves ahead and fills the volume between the boundaries  $\beta$  and  $\gamma$ . The resulting concentration,  $n_s$ , of the separated nucleic acid is thus the sum of the concentration,  $\rho n_f$ , due to the free acid in the body of the mixture and that arising from the decomposition of the complex. The factor  $\rho$ , it will be recalled, corrects for the dilution in the  $\delta$  boundary. The protein  $P$  resulting from the partial dissociation of the complex,  $X$ , accumulates in the volume  $V_\beta$

<sup>13</sup> It may be noted that in all of the cases considered here, with the possible exception of the fourth case, a qualitative symmetry is retained by the patterns insofar as the same number of boundaries, exclusive of the  $\delta$  and  $\epsilon$  effects, is present in both channels. In some mixtures, however, even this type of symmetry is not observed. Thus Chargaff, Ziff, and Moore (Chargaff, E., Ziff, M., and Moore, D. H., *J. Biol. Chem.*, 1941, **139**, 383), in their electrophoretic studies of serum albumin-heparin mixtures, frequently observed three boundaries in one channel and two in the other. We have made similar observations with mixtures of ovomucoid and nucleic acid. Asymmetries of this type have not been satisfactorily explained but are, possibly, a reflection of the known complexity of whole serum albumin (McMeekin, T. L., *J. Am. Chem. Soc.*, 1940, **62**, 3393) on the one hand and ovomucoid on the other (cf. footnote 2).

at the concentration  $\rho p_f$ . Due to the dissociation occurring in the boundary at  $\beta$ , the displacement,  $V_\beta$ , of this boundary is proportional to neither  $u_x$  nor  $u_P$  but has an intermediate value.

In the cathode side of the channel the nucleic acid,  $N$ , instead of escaping from the mixture, migrates into it. The complex thus tends to find itself in a region devoid of  $N$  and dissociates in the boundary at  $c$ , maintaining the equilibrium. The displacement,  $V_c$ , of this boundary is again proportional to neither  $u_N$  nor  $u_x$  but to an intermediate value. Pure  $P$  is left behind in the region between  $b$  and  $c$  at a concentration  $p_c$  which is greater than  $p_f$  but less than  $p_i$ . This material migrates with the normal mobility of  $P$  and hence the displacement  $V_b$  is proportional to  $u_P$ .

Much effort has been expended in an attempt to compute the mass action constant,  $K$ , of equation 4. For this computation from the electrophoretic data it is necessary to find a value of  $u_x$ ; *i.e.*, the mobility of the complex. However, as indicated above, the motion of the boundaries involved also includes an effect due to the decomposition of this complex, making direct determination of this quantity impossible.

#### SUMMARY

Electrophoretic patterns of mixtures of ovalbumin and yeast nucleic acid indicate that the constituents migrate independently of each other in buffer solutions of 0.1 ionic strength and at pH values somewhat higher than the isoelectric point of the protein. In the isoelectric region, however, the patterns from the two sides of the channel exhibit asymmetries that can be explained by assuming the existence in the mixture of appreciable concentrations of a reversibly dissociable complex between the components. Formation of this complex is favored by increasing concentrations of the components and decreasing ionic strength. At pH values below the isoelectric point partial precipitation of the complex occurs.

The patterns obtained from each side of the channel in the electrophoresis of a mixture of two components, which form a dissociable complex, indicate only two boundaries, aside from the  $\delta$  and  $\epsilon$  effects. One of these is a normal boundary whose displacement is proportional to the mobility of a component that has separated from the mixture. In the other boundary, however, dissociation of the complex occurs and consequently the displacement of this boundary corresponds to the mobility of neither component nor to that of the complex. Moreover, the areas under the refractive index gradient curves are not proportional to the stoichiometric concentrations of the components. However, equations are developed with the aid of which an electrophoretic analysis of the mixture is possible. This analysis requires the use of data from the patterns of both channels.

# A CYCLOSCOPIC STUDY OF THE HUMAN ELECTROENCEPHALOGRAM\*

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The object of this report is to introduce the successful application of a relatively new and rapid wave analyzing instrument to the study of the potential variations derived from the human brain. This instrument is called a *cycloscope*. It was invented by Dr. A. E. Douglass (1), Director Emeritus of the Steward Astronomical Observatory and Director of the Tree-Ring Laboratory of the University of Arizona.

The cycloscope has proved to be a powerful instrument in the study of events occurring in impermanent cycles; that is, in short sequence, quasi-periodic phenomena. It has given rise to important results in the field of meteorology, in the terrestrial correlates of sunspot cycles, and allied fields.

The human electroencephalogram shows a characteristically quasi-periodic behavior. This property of the electroencephalogram is manifested by fluctuations, around a fairly constant mean value, in the time elapsed (wave length) between successive maxima or minima of the potential variations.

Together with the foregoing characteristic the wave patterns observed in the electroencephalogram often show the presence of random frequencies and volleys of variable sequence cyclic activity.

Since the cycloscope was invented primarily for the study of just such wave phenomena as exhibited by the electroencephalogram it was decided to investigate: (1) the degree of permanency of the basic electroencephalogram pattern in various individuals; (2) the presence of cycles other than those which can be recognized by unaided visual means.

Throughout this discussion a *cycle* will indicate a significant *sequence* of waves that tend to fluctuate in length about a mean value, seldom varying from it by more than one or two tenths.

## *Method and Description of Apparatus*

The cycloscope operates on optico-mechanical principles. The essential components of the apparatus are illustrated in Fig. 1.

\* This work is in part aided by the Supreme Council 33° Scottish Rite, Masons of the Northern Jurisdiction, U. S. A.

The waves to be analyzed are transferred by pantograph, or other suitable instrument, to a strip of heavy opaque paper. A base-line, known as a cutting line, is then drawn through these transferred waves. The cutting line is made approximately at the height of the mean minimum, and wherever possible this line is kept parallel to the horizontal axis. The maxima isolated by the base-line are cut-out and the resultant perforated strip is called a *cycleplot*.

The light transmitted through the wave maxima (cut-outs) is obtained from a bank of ordinary incandescent lamps behind a diffusing ground glass screen. The

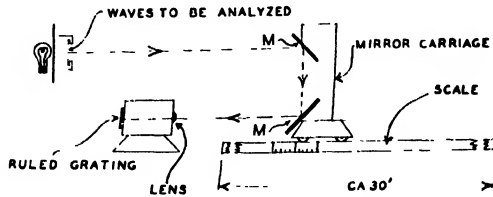


FIG. 1. Schema of cycloscope. Modified from Fig. 22a in Douglass' article (1).

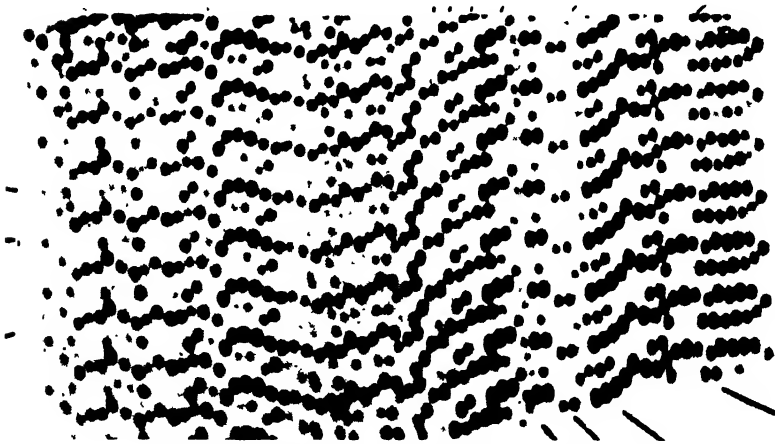


FIG. 2. Plate 24 in Douglass' article (1).

light rays passing through the cut-out maxima are made to fall on a mirror (*M*) placed in the optical axis at an angle of  $45^\circ$ . This diverts the ray normal to its original path; the diverted ray impinges on a second  $45^\circ$  mirror set in the axis of this light path, and thence back through the analyzing elements.

The analyzing elements consist essentially of a cylindrical lens with vertical axis and a grating, or analyzing plate. The cylindrical lens elongates the light ray bundles transmitted through the openings in the cycleplot. From the inherent character of the vertical cylindrical lens the time axis (horizontal) is reduced in scale but is not distorted. The elongated images of the maxima are made to fall on the grating (constant 0.02 inch) which is mounted at an angle approximately  $17^\circ$  to the vertical. The grating thus segments the image formed by the optical system. The presence and dominance of cycle lengths is manifested by both the character of the alignment

and the intensity of the light segments; different cycle lengths show at any given setting of the mirrors different angular alignments with respect to the horizontal axis.

The scale of cycle lengths is calibrated in such a way that one may read the cycle length directly when there is a horizontal alignment of light segments and the original plot is on a standard horizontal scale.

In actual operation the angularly arrayed light segments are rotated into the horizontal by increasing or decreasing the object-mirror distance. The cycle length as read directly from the scale may be corrected by a proper factor if the scale of the cycloplot is not standard.

Fig. 2 is an excellent illustration of what one sees when looking through the eyepiece of the cycloscope. (The illustration represents a cyclogram of the light curve of the variable star SS Cygni.)

### RESULTS

By means of the cycloscope excerpts from 18 electroencephalographic records were analyzed at Tucson by Edmund Schulman of the University of Arizona. Cycleplots were prepared under his direction by the author and differentiated only by number, so that the cycle analysis was as objective as possible. The list of cycles thus obtained formed the basis for the following study.

The analytic study was made on records obtained from the following clinical groups; normal (5), dementia praecox (6), epileptic (5), and "organic" brain disease (2). In this discussion only the midline fronto-occipital derivations will be considered.

Fig. 3 shows the cycloscopically determined distribution of cycles (or frequencies) present in two records, together with the waves actually analyzed. The waves shown were photographed from a cathode ray oscilloscope by means of a continuously moving camera of our own construction and design. The thermionic amplifier used was constructed in our own laboratory. The frequency response of this amplifier and recording system is flat from 2 to 200 cycles per second.

In this diagram the cycle strength, which is a function of the alignment and intensity of the light dots, is plotted against frequency in cycles per second. The fractional values of the frequencies are disregarded in this preliminary report.

In part A of Fig. 3 one sees the distribution of the various frequencies obtained from a presumably normal subject. It is evident that this individual shows a dominant rhythmic alpha pattern in the range of 8 to 10 waves per second. Furthermore, it is observed that contrary to the impression given by the accompanying record, there are at least *four definite different cycles present*.

Part B of Fig. 3 shows the frequency distribution in a subject with the clinical diagnosis of dementia praecox. It is clearly seen that there tends to be a spread of cycle lengths as compared to that of the "normal."



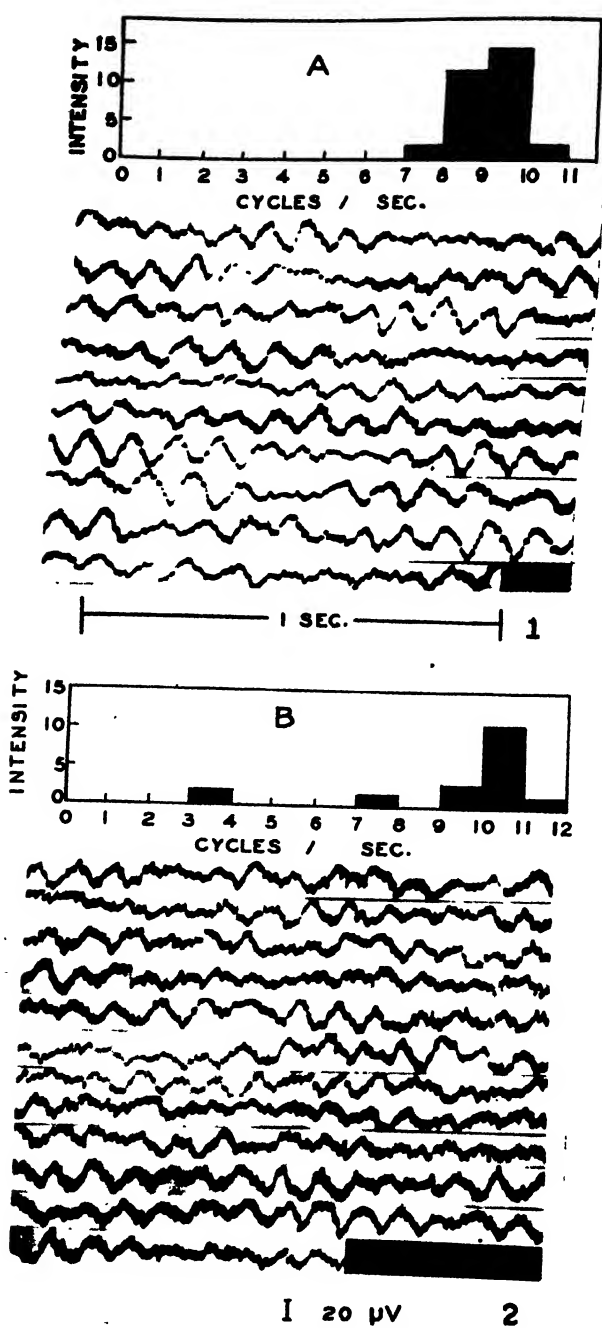


FIG. 3

The distinction between normals and dementia praecox as brought out by this cycle analysis is not as definite and clear cut as would appear from these two examples. In the entire group of records analyzed there is an *indefinite* boundary between normals and schizophrenics.

Fig. 4A represents the cycloscopic analysis of a record taken during a petit mal epileptic attack. There is seen to be a definite shift of the cycle distribution to a lower frequency range. The electroencephalographic recordings of Fig. 4 are made with an A.M. Grass ink-writing instrument.

Fig. 4B gives the analysis of a record from an individual with atypical epileptiform attacks. One observes that little or no dominant frequency is found by the analytic method employed.

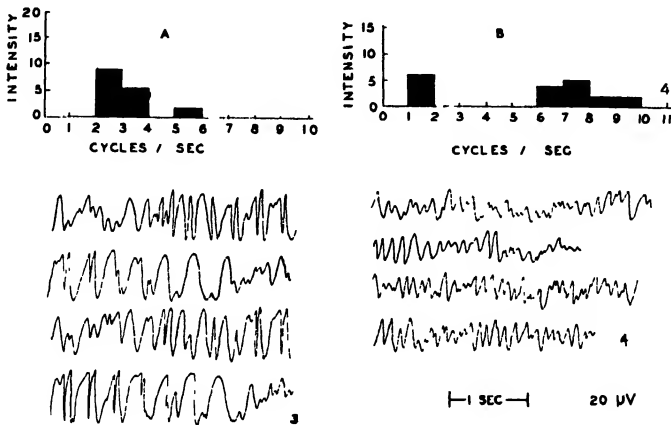


FIG. 4

The frequency distribution in this last case is especially interesting in that we observed it in two other cases of atypical epilepsy.

The records associated with "organic" brain disease showed the closest frequency distribution of the entire series. An 8 per second wave pattern completely dominated the tracings. Other cycles in these two records were negligible.

The cycloscope not only is capable of determining the basic frequencies and cycles present in a given sample of record, but it can quantitatively describe other important properties of the wave pattern, such as the phase relationships of the successive waves comprising the sequence, and the mean amplitude of the wave sequences.

Phase changes in some of the records analyzed have been observed. The physiologic concomitants of this phenomenon or its importance must await further study.

## CONCLUSIONS

Cycloscopic analyses of a series of electroencephalograms obtained from a mixed group of individuals, clinically classified as normal, schizophrenic, and "frank" epileptic, show the following characteristics:

1. A rather closely regulated potential oscillation which remains predominant throughout the sample of record under study.
2. Associated with this dominant cycle other definite anharmonic cycles are clearly evident. These associated cycles may operate in intermittent sequences or simultaneously with the dominant cycle.

Subjects with "atypical" epilepsy show an apparently characteristic spread of low intensity cycles.

## REFERENCE

1. Douglass, A. E., 1936, Climatic cycles and tree growth, *Carnegie Institution of Washington, Pub. No. 289*, 3.

## QUANTITATIVE CHEMICAL STUDIES ON HEMOLYSINS

### I. THE ESTIMATION OF TOTAL ANTIBODY IN ANTISERA TO SHEEP ERYTHROCYTES AND STROMATA\*

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(Received for publication, December 2, 1941)

Hemolysin in antisera is customarily estimated by "titer" and this measurement has served its purpose in the evaluation of antisera and in the determination of the relative degree of sensitization of erythrocytes. However, knowledge of the mode of action of hemolysins on red cells and of the quantitative relations of hemolysins to erythrocytes and complement has been difficult of access not only because of the complicated and sensitive nature of the reactions and their components, but also because methods of assay yielding end "titers" are, taken alone, without significance as to the actual weights of the reactants. Moreover, these methods do not even give relative values when different substances or functions are measured by end "titers," since the actual quantity of one substance required to produce its characteristic effect may greatly exceed that of another substance producing the same or a different effect. End "titers" are often, therefore, in opposite ratio to the actual quantities involved. Examples are the low "titers" of antipneumococcus sera of high antibody content as contrasted with the high "titers" of anti-*Salmonella* sera of low antibody content, and the lower "titer" but apparently higher content of first component in complement as compared with the higher "titer" but lower content of second and fourth components (1, 2). "Titers," then, depend upon the relative proportions in which the components of a given system interact, as well as upon the absolute quantities present, and are only interpretable, on passing from one reacting system to another, when reacting proportions as well as quantities are known. To return to the hemolytic system, since only "titers" of the components could be estimated in an effort to determine the very quantities which would necessarily have to be known before "titers" could be interpreted, lack of theoretical progress in this field is readily understandable.

The present communication deals with an attempt at the estimation of hemolysin in absolute, or weight units. Data of this kind would not only be useful in studying the quantitative relations between hemolysin, the red cell,

\* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

and complement, but would also supply a factor by which hemolysin "titers" could be converted into weight units. While this objective has not yet been attained, it has been possible to estimate total antibody in hemolytic antisera with a fair degree of accuracy and to use this value as a first approximation in the study of the relations sought (3).

The amount of nitrogen in the hemolytic unit has been estimated by Locke, Main, and Hirsch (4), by Brunius (5), and by Chow and Zia (6). The reasons for the large discrepancies in the estimates reached in the first two investigations are again (*cf.* 3) discussed below, together with an evaluation of the method used by Chow and Zia.

#### EXPERIMENTAL

##### *Materials and Methods*

*Sheep Cell Stromata.*—Lot 1: Three liters of sheep blood were defibrinated with glass beads immediately after bleeding, filtered through cloth, diluted with 4 volumes of saline, and centrifuged at room temperature in a Sharples centrifuge. The deposit of red cells was laked with 20 volumes of distilled water and centrifuged as before. The stromata were washed twice with water and twice with 0.9 per cent saline at room temperature, followed by two more washings with saline at 0°C.<sup>1</sup> The pale brown stromata were resuspended in saline containing 1:10,000 merthiolate.<sup>2</sup> The suspension was kept in the ice box and portions were washed at 0° before use.

Lots 2 and 3 were prepared similarly except that all operations, including the rather tedious process of centrifugation,<sup>1</sup> were carried out in the cold.

A portion of Lot 2 was heated at 56°C. for 40 minutes (7) (heated suspension).

*Antisera.*—Rabbits were injected intravenously with stromata suspensions (Lots 1 and 2) containing 0.5 mg. N per ml. The first course consisted of 13 injections averaging 0.75 ml. The second and third courses consisted of 10 and 12 injections respectively, of an average of 1.5 ml. of suspension. The fourth course comprised 10 injections and the fifth 11 injections of an average of 1.5 ml. of suspension (rabbits 7.62, 7.66). Rabbit 7.60 received only the first, third, and fifth courses. Rabbit 7.83 was injected subcutaneously instead of intravenously, receiving one course of 7 injections of 0.5 ml. followed by a month's rest, and a second course of 3 injections of 0.5 ml., 2 of 1 ml., 2 of 1.5 ml., and 4 of 2 ml. of suspension. A number of commercial antisera to sheep red cells were also used.<sup>3</sup>

*Analytical Methods.*—All antisera were inactivated at 56°C. for 50 minutes (thermometer in serum) (2), neutralized to pH 6.7–7.0 (glass electrode), and centrifuged in the cold shortly before use. Duplicate 1.0 ml. portions of undiluted antiserum, 2.0 ml. of saline, and 1.0 ml. of the stromata suspension were mixed at 0°C. Duplicate blanks were also run on 1.0 ml. of serum alone and with 1.0 ml. of stromata and 3.0 ml. saline alone. The tubes were allowed to stand in the ice box for 48 hours, with occasional mixing of the contents. The stromata usually showed pronounced agglutination in the presence of antiserum.

<sup>1</sup> In a refrigerated centrifuge manufactured by the International Equipment Company, Boston.

<sup>2</sup> Manufactured by Eli Lilly and Company, Indianapolis.

<sup>3</sup> Kindly supplied by E. R. Squibb and Sons, Inc., and by the Lederle Laboratories.

The determination of the antibody N precipitated was similar to that of the quantitative agglutinin procedure (8-10). The tubes were centrifuged in the cold and the precipitates washed three times with 0.9 per cent saline at 0°. The supernatants of duplicate determinations were combined and recentrifuged, and any deposits left were washed with the saline washings poured from the main tubes and finally recombined with the main precipitates. The initial supernatants, after the recentrifugation, were again set up with 1.0 ml. of stromata suspension and new blanks. The precipitates and the recentrifuged supernatants and saline washings from all stromata blanks were analyzed separately. The solutions always contained a small amount of N (about 0.04 mg.) due to soluble material remaining in the stromata in spite of the repeated preliminary washings.

Antibody N in the tubes containing serum and stromata was taken as the difference between the total N precipitated and the stromata N deposited in the stromata blank tubes after three washings and centrifugations. The antibody values obtained are probably slightly too high because of the antigen which dissolved. Correction would be difficult since it is not certain how much of the dissolved N represented inert material, or whether the same amount of N was washed out of the stromata which had combined with antibody.

*Hemolysin Titers.*—The hemolytic titers were determined from the dilution at which the sera would just sufficiently sensitize an equal volume of a 5 per cent suspension of washed sheep red cells to give complete hemolysis in 20 minutes at 37°C. in the presence of 2 units of complement.

Data obtained as above on a dilution of serum 7.66<sub>s</sub> are given in Table I. Antibody N data for a number of sera are correlated with hemolytic titers in Table II.

*Effect of Experimental Conditions on Antibody Precipitation.*—(a) *Hydrogen Ion Concentration:* A dilution of serum 7.66 with a hemolysin titer of 1200 was absorbed with stromata after adjustment of one portion to pH 6.0 and another to pH 8.0 (glass electrode). The pH was redetermined after each absorption and slight readjustments to the original values were necessary for the first two. The total amounts of antibody N removed in three absorptions at low pH were 0.26, 0.01, and 0.02 mg. N, with a total of 0.29 mg. N, and in the more alkaline series, 0.25, 0.03, and 0.02 mg. N, with a total of 0.30 mg. N per ml. The fourth absorptions were negative in both cases. Hemolytic titers in both series were reduced to less than 6 units per ml. There were thus no differences in behavior in the absorptions at these two hydrogen ion concentrations and the precaution of neutralization, taken in most of these studies, would therefore seem unnecessary.

(b) *Effect of Temperature:* 1.0 ml. of undiluted rabbit serum 40H301 and 1.0 ml. of a 1:1 diluted donkey antiserum 186H102 to sheep red cells were each absorbed with stromata in parallel series at 0° and 37°C. The data are given in Table III. It will be noted that almost as much antibody N was removed at 37° as at 0°C.

(c) *Effect of Heat on Antigen Suspension:* Absorptions were made with heated and unheated suspensions of stromata. First bleedings from rabbits

TABLE I  
*Estimation of Antibody in Anti-Sheep Cell Rabbit Hemolysin by Addition of Sheep Cell Stromata;  
1.0 Ml. Serum, 0°C.*

Absorption	Stromata N used*	Total N precipitated	Antibody N found (mean)	Calculated to 1.0 ml. of original serum
	mg.	mg.	mg.	mg.
1st	$\begin{cases} 0.718 \\ 0.710 \end{cases}$	$\begin{cases} 0.968 \\ 0.978 \end{cases}$	0.264	0.26
2nd†	$\begin{cases} 0.488 \\ 0.478 \\ 0.472 \end{cases}$	0.492‡	0.012	0.01
3rd§	$\begin{cases} 0.486 \\ 0.484 \\ 0.492 \end{cases}$	0.530§	0.042	0.02
4th§	$\begin{cases} 0.488 \\ 0.478 \\ 0.480 \end{cases}$	0.486§	<0.01	0
Total.....				0.29

\* Values for washed stromata blanks, in duplicate or triplicate.

† On combined supernatants from first absorption.

§ On supernatant of preceding absorption.

TABLE II  
*Absorption of Antibody in Anti-Sheep Cell Hemolysin by Sheep Stromata Suspensions, per  
1.0 Ml. Undiluted Serum, 0°C.*

Serum	Hemo-lytic titer*	1st absorption		2nd absorption		3rd absorption		Total antibody N	Hemo-lytic titer + antibody N
		Antibody N	Hemo-lytic titer	Antibody N	Hemo-lytic titer	Antibody N	Hemo-lytic titer		
		mg.		mg.		mg.		mg.	
L <sub>1</sub>	1,200	0.24	<30	0.05	<4	0		0.29	4,100
L <sub>2</sub>	2,500	0.11	≤120	0	<6			0.11	23,000
L <sub>3</sub> †	3,000	0.71	>240	0.11	>240	0	100	0.82	3,500
L <sub>4</sub>	2,000	0.48	<24	0.03	<12			0.51	3,900
S <sub>1</sub>	1,200	0.36	130	0.10	36	§		0.46	2,600
S <sub>2</sub>	750	0.20	60	0.02	40	0.02	24	0.24	3,000
7.60 <sub>4</sub>	600	0.18	<15	0.07	<5	0.02		0.27	2,200
7.62 <sub>4</sub>	1,400	0.24	15	0.08	<5	0.01		0.33	4,200
7.66 <sub>4</sub>	1,700	0.29	15	0.09	<5	0.02		0.40	4,300
7.83 <sub>  </sub>	10	0.09	0	0.01	0	0		0.10	100

\* The titrations given are the reciprocals of the dilution at which the sera completely hemolyzed an equal volume of 5 per cent sheep red cell suspension in the presence of two units of guinea pig complement and are thus independent of the volumes chosen so long as this relation to the red cells is maintained. The antibody N estimations are all calculated to 1.0 ml. of the undiluted serum.

† Pony serum.

§ Determination lost.

|| Rabbit injected subcutaneously with stromata.

injected with unheated stromata were used (Table IV). Although the total amounts of antibody N removed by heated suspensions approached those taken

TABLE III

*Estimation of Antibody in Anti-Sheep Cell Hemolysins by Stromata Suspensions at 37° and 0°C., per 1.0 Ml. Serum*

Serum and temperature	Hemolytic titer	1st absorption		2nd absorption		3rd absorption		Total antibody N
		Antibody N	Hemolytic titer	Antibody N	Hemolytic titer	Antibody N	Hemolytic titer	
		mg.		mg.		mg.		mg.
L <sub>6</sub> , 37°	1,600	0.26	200	0.06	90	0.03	50	0.40*
L <sub>6</sub> , 0°	1,600	0.29	400	0.10	<225	0.04	<25	0.43
L <sub>6</sub> , † 37°	2,400	0.27	1,600	0.06	270	0.04	250	0.37
L <sub>6</sub> , † 0°	2,400	0.28	1,000	0.09	<225	0.05	50	0.42

Approximately equal, but accurately measured, quantities of stromata were used for each absorption.

\* Includes 0.05 mg. N obtained on fourth and fifth absorptions. Final hemolytic titer 30

† Donkey serum. Determinations made and reported on basis of 1:1 diluted serum.

TABLE IV

*Absorption of Antibody in Anti-Sheep Stromata Rabbit Hemolysins by Heated and Unheated Sheep Stromata, per 1.0 Ml. Serum, 0°C.*

Serum	Hemolytic titer	1st absorption		2nd absorption		Total antibody N
		Antibody N	Hemolytic titer	Antibody N	Hemolytic titer	
		mg.		mg.		mg.
Sera absorbed with heated stromata suspension						
7.60 <sub>1</sub>	<100	0.06	>1	0.02	<8	0.08
7.62 <sub>1</sub>	>1,000	0.09	ca. 500	0.05	>200	0.20*
7.66 <sub>1</sub>	<1,000	0.08	ca. 300	0.02	ca. 10	0.13†
7.57 <sub>1</sub>	<1,000	0.09	>500	0.04	>64	0.17‡
	>500					
Sera absorbed with same suspension, unheated						
7.60 <sub>1</sub>	<100	0.09	0			0.09
7.62 <sub>1</sub>	>1,000	0.22	75			0.22
7.66 <sub>1</sub>	<1,000	0.17	<24			0.17
7.57 <sub>1</sub>	<1,000	0.19	0			0.19
	>500					

\* Includes 0.06 mg. N from third and fourth absorptions.

† " 0.03 " " " a third absorption.

‡ " 0.04 " " " " " " "

out by unheated stromata, the unheated stromata suspension was the more active antigen per milligram of N used. 0.50 mg. N of the unheated suspension precipitated the same amount of antibody N in one absorption as did 2.99 mg. N of heated stromata in four absorptions. The data are summarized in Table IV.



*Combining Proportions of Stromata and Antibody.*—The existence of more than one combining ratio between stromata and antibody is indicated. For example, 1.0 ml. portions of a 1:1.5 dilution of serum 7.66<sub>4</sub> were set up in duplicate with 1.0, 1.5, and 2.5 ml. of stromata suspension in a total volume of 8.0 ml.

Stromata N added	1st absorption	2nd absorption*	Total antibody N precipitated
	Antibody N precipitated	Antibody N precipitated	
mg.	mg.	mg.	mg.
0.476	0.23	0.04	0.27
0.714	0.24	0.03	0.27
1.188	0.29	0.01	0.30

\* On pooled supernatants. Antibody N given is one-half of that found on addition of 0.476 mg. of stromata N in each case.

The smallest amount of antigen used removed practically as much antibody N as did a quantity 50 per cent larger, although neither completely exhausted the serum. Other particulate antigens, such as specific precipitates (10), show a similar behavior in their homologous antisera; the slope of the line representing antibody N/antigen, plotted against antigen N added, is usually much less than with soluble antigens.

*Removal of Complement by Stromata-Antibody Precipitation.*—In (3) it was mentioned that the combining component(s) of complement was taken up in the reaction of sheep cell stromata with hemolysin. The data upon which this statement was based are given in the protocol. The tubes were allowed to stand 2 hours at room temperature, after which they were centrifuged and washed three times with 2.5 ml. saline in the cold (*cf.* 2).

No. of tubes . . . . .	2	2	3	3	3
Guinea pig serum, C', ml. . . . .	3.0	3.0			3.0
Inactivated* guinea pig serum, iC', ml. . . . .				3.0	
Rabbit hemolysin, ml. . . . .	1.0		1.0	1.0	1.0
Sheep cell stromata, ml. . . . .		1.0	1.0	1.0	1.0
Saline, ml. . . . .	4	4	6	3	3
Total N precipitated, mg. . . . .	{ 0	{ 0.410	{ 0.504	{ 0.524	{ 0.602
	{ 0	{ 0.382	{ 0.508	{ 0.520	{ 0.606
			{ 0.514	{ 0.504	{ 0.610
Mean, mg. . . . .	0	0.396	0.509	0.516	0.606
Stromata N to be subtracted (column 3), mg. . . . .			0.396		
Hemolysin N precipitated, mg. . . . .			0.11		
iC' to be subtracted, mg. . . . .					0.516
C' N precipitated, mg. . . . .					0.09

Hemolytic units of C' taken 285.

" " " " remaining <1.

\* At 56°C. for 50 minutes. The symbols used are explained in (2).

The quantity of C' N removed is of the same order as that taken out by the specific precipitates (2). An estimation with S III-rabbit anti-S III at the same time gave 0.07 mg. of C' N.

#### DISCUSSION

Attempts have previously been made, by purification and isolation of the antibody, to define a weight unit for hemolysin. Locke, Main, and Hirsch (4) purified rabbit anti-sheep cell hemolysin and found 0.007  $\gamma$  of nitrogen per hemolytic unit in their best preparation. Brunius (5), on the other hand, using the Forssman antigen for selective extraction of the hemolysin, found only 0.0002  $\gamma$  of N in a smaller hemolytic unit. In both laboratories the preparations isolated usually contained ten times as much N per unit. As already pointed out (3) Brunius' value probably represents the actual hemolytic antibody unit more closely than Locke, Main, and Hirsch's figure, or that now reported, 0.03  $\gamma$  of N.<sup>4</sup> The latter, however, have the advantage of giving an insight into the actual weight of antibody deposited on the red cell from rabbit antisera.

These isolation techniques (4, 5) are naturally too laborious for analytical purposes. A method for the estimation of hemolysin in weight units has recently been described by Chow and Zia (6), who agglutinated sheep red cells with a measured volume of hemolysin, washed the deposit, and subsequently removed the hemoglobin by laking. The amounts of antibody found greatly exceeded those indicated in the present study, possibly because hemoglobin and other soluble products in the blanks without antiserum would be more easily washed out than from cells on which antibody had been deposited.

The method now proposed is a modification of the quantitative agglutination procedure (8-10) and depends upon the addition of antibody to sheep cell stromata from which as much as possible of the soluble components has been removed. Comparison of hemolytic titers of antisera before and after successive absorptions with stromata (Table II) shows that hemolysins are removed, as had been found by previous workers. Reproducible results are readily obtained, so that the method may serve, as a first approximation, for the estimation of hemolysin in weight units.

However, two factors combine to limit the utility of the method for its original object. One, of relatively minor consequence, tends to make the results somewhat too high. In spite of the pains taken to wash the stromata used small quantities of soluble material (about 0.04 mg. of N) were invariably found in the washings from the blank tubes. At least a portion of this material gave precipitin reactions in the antisera, but since it is uncertain whether or not similar amounts were washed out of the stromata-antiserum residues no correction has been made for this effect. It is therefore possible that the antibody N values found are too high by all or a portion of the N washed out of the con-

<sup>4</sup> Calculated from the figures in Table II for 0.1 ml. of hemolysin dilution.

trols. Stromata heated after the washing process according to Landsteiner and van der Scheer's method for red cells (7) are more easily centrifuged and appear to be slightly less soluble, but they cannot be used conveniently for the quantitative absorption of antibody as their antibody-binding capacity is too low (Table IV).

The second limiting factor is that the method, strictly speaking, is one for the estimation, not of hemolysin, but of total antibody in the hemolytic antisera. Sheep red cells and even washed sheep cell stromata are complex collections of antigens and only one or a limited number of these, presumably gives rise to true hemolysins. The remaining antibodies stimulated by injection of cells or stromata into rabbits add to the stromata suspension when this is mixed with antiserum, or to red cells, but probably take little part in hemolysis. This is probably the chief reason for the discrepancy in the values given (4, 5) for the quantity of N in the hemolytic unit. According to these values (p. 529) the antibody N figures may exceed true hemolysin N by a factor of 10 to 100, although these values are not to be taken too literally on account of the different units and end points compared. In spite of this, there does appear to be a fair proportionality between total antibody N content and hemolysin titer in rabbit anti-sheep hemolysin, as already noted by Chow and Zia (6) although on a different numerical basis. If one excludes the pony and donkey sera because of species differences, the serum of the subcutaneously injected rabbit and one other commercial rabbit serum, L<sub>2</sub>, that was entirely out of line, ten anti-sheep cell and anti-stromata rabbit sera showed a titer: antibody N ratio of  $3500 \pm 600$ . In other words, an antiserum which, at a dilution of 1:3500, just completely hemolyzes an equal volume of 5 per cent sheep red cell suspension in the presence of 2 units of complement, should contain, within about 20 per cent, 1 mg. of antibody N per ml. None of the sera examined contained as much antibody N as this in a single milliliter. On this basis it might be more reasonable, both from the standpoint of purveyor and purchaser, to sell and buy hemolytic antisera by the milligram of total antibody nitrogen, rather than on the basis of units which might vary considerably from laboratory to laboratory and which provide no inkling of the actual antibody content.

If data obtained according to the new method are to be used in a consideration of the relation between hemolysin, red cells, and complement in the process of hemolysis, it must be remembered that the antibody N values are maximal and can only be used as hemolysin N as a first approximation, subject to correction by a factor still to be determined. This was emphasized in attempts to trace this relationship in (3). As there pointed out, the total antibody N value is of use in an understanding of the hemolytic process as actually carried out, for all of the antibody combines with the red cells present and also plays a major part in the fixation of complement, as shown in protocol 2 on p. 528.

Efforts are now being made to estimate accurately the actual amounts of hemolysin present in these antisera.

#### SUMMARY

1. Total antibody in hemolysins may be estimated from the nitrogen added to sheep stromata suspensions.
2. The method is applied to a number of hemolysins and a correlation, valid to within 20 per cent, established between hemolytic titer and total antibody.
3. When stromata combine with antibody in the presence of guinea pig complement they may take up at least 80 per cent of their weight of complement combining component(s).

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# THE MECHANISMS OF X-RAY EFFECTS ON CELLS\*

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Injury or death of cells subjected to x-rays has been attributed to various physiological effects such as changes in metabolic rate, alterations in cytoplasmic viscosity and permeability, or the effect on enzymes (1). Scott concludes that these physiological effects of x-rays are of secondary importance since very high doses are required to produce significant effects. The "primary" effect of radiation on dividing somatic cells does appear to be caused by physiological changes, but this effect is temporary (2). Irradiation of meiotic cells of Orthoptera appears to produce no direct effect on the chromosomes and fragmentation does not occur until the nuclear membrane is broken down. This action is attributed to chemical changes induced in the cytoplasm (3). The production of chromosomal aberrations by x-rays may be delayed for an hour in *Tradescantia* (4) or for several weeks following irradiation of *Drosophila* sperm (5). This delayed action has led to the suggestion that these x-ray effects are indirect (6).

Most of the recent work on x-ray effects supports the hypothesis of "direct hit" action. Henshaw (7) has shown that the lethal action of x-rays is produced by the direct effect on the cell nucleus. In both *Tradescantia* (4) and *Drosophila* (5) the dosage curves for chromosomal aberrations support the "direct hit" mechanism of x-ray action. This theory also is supported by time-intensity experiments (8) and by the differential action of x-rays and neutrons (9, 10). These experiments are limited to x-ray effects on single cells.

The relative rôle of indirect physiological effects and of the "direct hit" mechanisms of x-ray action can be analysed by determining the effect of irradiation on subsequent sensitivity. If x-rays produce chemical changes which persist for a relatively long time, or initiate chromosome alterations which are effective in producing aberrations only at later stages in the nuclear cycle, then the effects of subsequent irradiation should be influenced by the previous irradiation.

The specificity of the types of chromosomal aberrations in *Tradescantia* microspores provides an opportunity for further analysis of x-ray effects on

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subsequent sensitivity. The duration of the nuclear cycle for tetrad formation to metaphase in the microspore is about 1 week. The chromosomes are in the resting stage for 5 or 6 days after meiosis and during this time x-rays produce alterations involving entire chromosomes. Cells rayed at prophase produce chromatid aberrations in which each sister chromatid behaves as an independent unit. The chromosome and chromatid aberrations can be differentiated readily (8).

Two series of *Tradescantia* buds were irradiated. Series 1 was rayed during the resting stage and again at prophase 2 days later. The x-ray doses were 300 r and 150 r respectively. Series 2 was rayed only at prophase simultaneously with series 1 which received 150 r. The experiment was repeated several weeks later using doses of 200 r and 100 r respectively. The cells were fixed 24 hours after the prophase irradiation. Those rayed both at the resting stage and at prophase showed both chromosome and chromatid aberrations, while

TABLE I

*Effect of Irradiation on Subsequent X-Ray Sensitivity of Tradescantia Microspores*

Series 1. Rayed at resting stage and again at prophase. Fixed 24 hrs. after second raying.

Series 2. Rayed only at prophase. Fixed 24 hrs. after raying.

	Series 1	Series 2
Total chromosomes.....	2556	4052
Chromosome breaks.....	154	0
Chromatid breaks.....	156 = 6.1 per cent	287 = 7.1 per cent

those rayed only at prophase had only chromatid aberrations at metaphase. The results of the two experiments were similar and the combined data are shown in Table I.

It is evident that previous irradiation did not increase prophase sensitivity as measured by the frequency of chromatid breaks. The percentage of chromatid breaks was somewhat less in the series previously rayed in the resting stage as compared with the aberrations produced by raying only at prophase, although the difference is of doubtful statistical significance.

These results are in accord with the fractional dosage experiments where the maximum rest periods were 4 hours (8). Evidently there is no physiological effect of x-rays which will increase the subsequent sensitivity of the microspores after a period of several hours or several days.

The slight decrease in aberration frequency of the successively rayed microspores suggested further analysis of chromatid aberrations produced in previously irradiated cells. Previous investigations have shown that breaks in the chromosomes are not at random for all loci, but are more numerous in the proximal end of the chromosome arms (11). This localization was attributed

to the rôle of the centromere in controlling mechanical stresses in the coiled chromonemata. If the frequency of illegitimate unions of broken ends of chromosomes is in some degree controlled by the centromere, their acentric chromosome fragments should be relatively resistant to x-ray effects. This assumption was tested by another series of successive exposures.

*Tradescantia* microspores were irradiated during the resting stage and 3 days later at prophase. The doses were 300 r and 150 r respectively. The cells were fixed 24 hours after the second exposure. A total of 11,502 chromosomes was examined. Among these were 681 dicentric and ring chromosomes resulting from irradiation at the resting stage. Each of these dicentric and ring chromosomes was accompanied by an acentric fragment. The average length of the acentric fragments was somewhat more than a normal chromosome arm since effective breaks are more frequent in the proximal ends of chromosome arms. But, to be conservative, we will assume that effective breaks are at random and that the 681 acentric fragments are equivalent to 340 normal chromosomes. We have then the equivalent of 11,152 centric chromosomes and 340 acentric chromosomes as a result of the first irradiation. The second exposure produced only chromatid aberrations, a total of 1215. Of these 1211 were in the centric chromosomes and only 4 were in the acentric fragments. If chromatid aberrations were at random we would expect 26 of them in the acentric fragments. It is apparent that effective breaks are much more frequent in the centric chromosomes than in the acentric fragments. The initial breaks must be at random, but those which are involved in chromosomal aberrations are in some way controlled by the centromere so that more illegitimate fusions occur in the centric than in the acentric chromosomes. This effect of the centromere could be attributed to polarity and spatial relations in the case of fusions between chromatids of different chromosomes, but the simple deletions involving only sister chromatids at a given locus also appear to be associated with mechanical stress which is related to the centromere. Some of the differential sensitivity to x-rays in centric and acentric chromosomes might be attributed to heterochromatic regions near the centromere. Kaufmann (12) has found that the heterochromatic loci in *Drosophila* chromosomes are especially sensitive to breakage by x-rays. In *Tradescantia*, however, the proximal ends of the chromosome arms are only about twice as sensitive as the distal ends. Although the distal ends of chromosome arms constitute a large proportion of the acentric fragments, the frequency of chromatid aberrations in acentric chromosome fragments is only about a ninth of that found in the centric chromosomes, when calculated on the basis of comparative length. The decreased sensitivity of acentric fragments would result in a lower frequency of chromatid aberrations in the microspores which had been irradiated previously at the resting stage. This conclusion is in accord with the trend shown in Table I, although the differences are not statistically significant.



The "direct hit" mechanism of x-ray action on cells is supported by the following observations. The relation between single chromosome breaks and x-ray dosage is linear, there is no threshold effect, and the frequency of simple deletions is independent of the time-intensity factor (13, 8). The frequency of chromosomal aberrations involving two breaks increases as the square of the dosage when x-ray intensity is held constant, but the exponent of the dosage curve approaches 1.0 as the x-ray intensity is decreased (8). Fractional dosage decreases the frequency of complex aberrations (14, 8). This behavior is attributed to restitution of broken chromosomes during the intervals between exposures, and the aberration frequency reaches a minimum when the rest periods are about an hour. Fractional dosage involving longer periods has no effect on subsequent sensitivity. Neutrons are more effective than x-rays in producing chromosomal aberrations (9) and in their lethal effect on bacteria (10). The increased effectiveness in both cases is attributed to the greater ionization density produced by the neutrons. The lethal action of radiation of bacteria is attributed to the production of lethal mutations. In the case of chromosome aberrations the lethal effect can be attributed either to chromosome deficiencies associated with chromosomal aberrations or to lethal mutations. Since Henshaw has shown that the lethal action of x-rays is produced by the direct effect on the cell nucleus, it seems probable that the chromosomes are involved.

Although the major effect of x-rays appears to involve chromosome alterations produced by "direct hits," there is evidence of a general physiological effect. It has long been known that x-rays produce a temporary cessation of nuclear activity (15). The "primary" effect of x-rays which results in chromosome clumping at metaphase also is attributed to physiological changes (2). These physiological effects are temporary and have a lethal effect only at very high doses. The production of alterations in meiotic cells of Orthoptera only when the nuclear membrane disintegrates at late prophase does suggest the production of some change in the cytoplasm (3), but the effect is similar to the primary effect of x-rays on dividing somatic cells.

The response of tissues to irradiation may involve more than the response of the individual cells, since injury to certain cells or physiological changes in the surrounding media may cause alterations which greatly change the behavior of cells not directly affected by irradiation. Such a response is indicated by the differences in x-ray sensitivity of different tissues or of different stages of the nuclear cycle (16). According to Shields Warren<sup>1</sup> many tumors become much more resistant to x-rays during intermittent treatment for several months. This decreased sensitivity seems to be caused by changed physiological conditions resulting from previous irradiation. Most of the lethal

<sup>1</sup> Unpublished data.

effect of x-rays on chick embryos is attributed to indirect action, and particularly to effects on the circulatory system (17).

The indirect physiological effects of x-rays also are found in irradiated onion seeds. If the germination of such seed is delayed, the chromosome aberration frequency is greatly increased (16). This effect appears to be comparable to the effect of age.

#### SUMMARY

Irradiation of *Tradescantia* microspores does not increase subsequent sensitivity to x-rays as measured by the frequency of induced chromosomal aberrations during the nuclear cycle. The slight decrease in sensitivity is to be expected because acentric fragments are less sensitive than the centric chromosomes. The physiological effects of x-rays appear to be of minor importance in causing injury or death of individual cells, and most of the deleterious effects can be attributed to "direct hits" which produce chromosomal alterations. In the reaction of tissues to x-rays the physiological effects may play a more important part.

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# THE RÔLE OF CARBONIC ANHYDRASE IN CERTAIN IONIC EXCHANGES INVOLVING THE ERYTHROCYTE\*

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There has been described elsewhere (1, 2) a permeability phenomenon analogous in certain respects to chemical catalysis but involving a diffusion process rather than a chemical reaction. It now appears that this phenomenon of "catalyzed diffusion," as it may be called for brevity, is of wider applicability than was at first suspected. The present paper deals with certain recently investigated aspects of this question.

The work had its origin in the observation of Ørskov (3, 4) that the rate of hemolysis of mammalian erythrocytes in solutions of ammonium chloride can be enormously increased—that is, 50 times or more—by the addition of a little bicarbonate. Ørskov in his first paper (3) suggested that carbonic acid has a specific effect of some sort on the erythrocyte which makes it more permeable to anions; later (4) he modified this view and postulated instead an increased permeability of the cell to the ammonium ion. For various reasons which have in part been set forth elsewhere (5) neither of these explanations seems to be satisfactory, and there has been proposed in their place the principle of catalyzed diffusion (2), in which the action of the bicarbonate is believed to be not primarily upon the cell at all but rather upon the solutions on the two sides of the cell membrane.

In Fig. 1 A is represented the mechanism (6) for the entrance of  $\text{NH}_4\text{Cl}$  into the erythrocyte, which in spite of certain objections (3, 4) that we believe to be answerable (5), seems best to explain the known facts. According to it  $\text{NH}_3$  molecules first enter the cell and there become converted into  $\text{NH}_4$  ions; a subsequent shift of anions through the anion-permeable membrane completes the process. The overall rate of transfer of the salt is slow, chiefly because of the extremely low concentration of  $\text{OH}'$  ions in the cell at any given time.

If, now,  $\text{NH}_4\text{HCO}_3$ —or any other bicarbonate—be added to the external solution, we have the conditions represented in Fig. 1 B in which a new molecule of very great penetrating power,  $\text{CO}_2$ , is present. Mass law considerations demand that for equilibrium the product  $[\text{NH}_4]_{\text{inside}} \times [\text{HCO}_3]_{\text{inside}}$  must

\* This work was made possible by a grant from The Rockefeller Foundation.

equal  $[\text{NH}_4]_{\text{outside}} \times [\text{HCO}_3]_{\text{outside}}$ . Since  $[\text{NH}_4]_{\text{outside}}$  is initially much higher than  $[\text{NH}_4]_{\text{inside}}$  because of the high external concentration of  $\text{NH}_4\text{Cl}$ , there will be a tendency to force  $[\text{HCO}_3]_{\text{inside}}$  above  $[\text{HCO}_3]_{\text{outside}}$ . This, however, in an anion-permeable cell would lead to an exchange of  $\text{HCO}_3'$  for  $\text{Cl}'$ . As a final result of these two processes  $\text{NH}_4\text{Cl}$  has entered the cell and

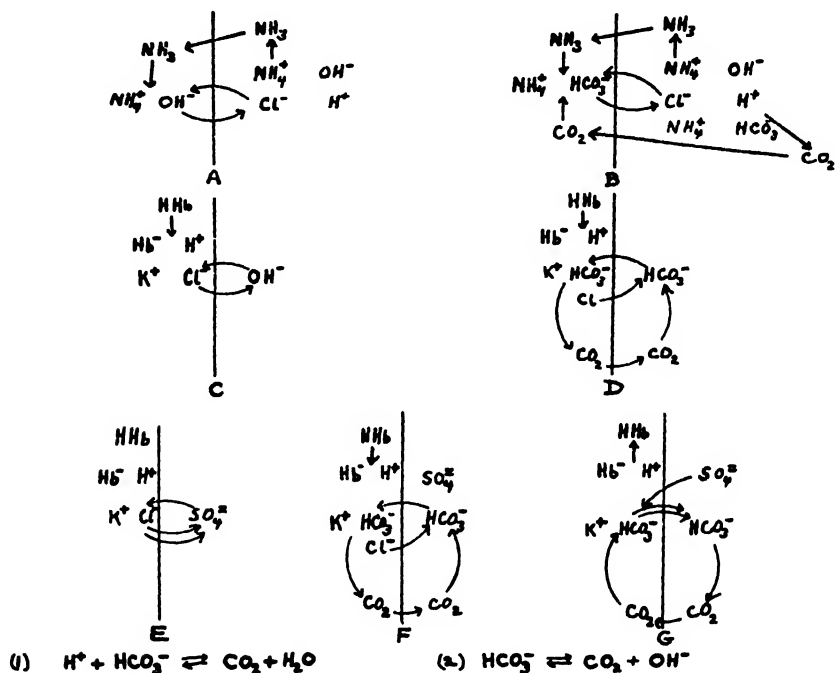


FIG. 1. Ionic exchanges involving the erythrocyte. The cell in each case is supposed to be on the left-hand side of the vertical line. (A) Cells in a solution of  $\text{NH}_4\text{Cl}$ . (B) Same, after the addition of  $\text{NH}_4\text{HCO}_3$ . (C) Cells in an alkaline bicarbonate-free solution. (D) Same, after the addition of bicarbonate. (E) Cells in a solution of a sulfate. (F) Same, after the addition of bicarbonate (first stage). (G) Same as F (second stage).

$\text{HCO}_3'$  is back again on the outside ready to repeat the cycle. It therefore serves as a catalyst, so to speak, to get the  $\text{NH}_4\text{Cl}$  into the cell more rapidly than it could enter by mechanism 1 A.

Experimental evidence for the cycle suggested in 1 B is provided by the action of certain narcotics which considerably decrease the permeability of the erythrocyte to anions, while having little effect on that to the molecules  $\text{CO}_2$  and  $\text{NH}_3$ . The form of the swelling curves in the presence of such substances is, in general, that demanded by the theory (2). Low concentrations of tannic acid may also be used to separate in part the "molecular" from the "ionic"

portions of the swelling processes. More direct evidence in favor of the catalyzed diffusion theory, however, is furnished by another method, entirely different in principle, with which the present paper is chiefly concerned.

## II

It will be noted that in the cycle represented in Fig. 1 B there is at one point a conversion of  $\text{CO}_2$  to  $\text{HCO}_3^-$ ; at another point the reverse change from

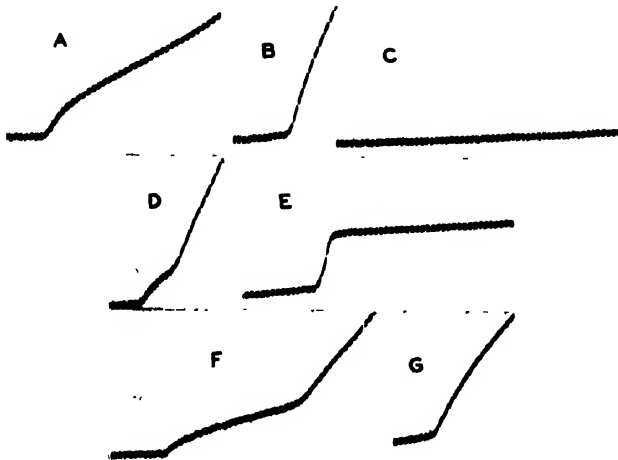


FIG. 2. Volume changes of washed beef erythrocytes in  $m/6$   $\text{NH}_4\text{Cl}$  to which  $\text{NH}_4\text{HCO}_3$  is added.  $22^\circ\text{C}$ . The breaks in the curves indicate intervals of 1 second. (A)  $0.003\text{ M}$   $\text{NH}_4\text{HCO}_3$  added. (B) Same, with carbonic anhydrase in the external solution. (C) Same as B in the presence of 125 mg. per cent of sulfanilamide. (D) Same as A, but carbonic anhydrase added 6 seconds after the bicarbonate. (E) Same as B, but  $\text{KCN}$  ( $0.007\text{ M}$ ) added a few seconds after the bicarbonate. (F) Same as D, but with half as much bicarbonate; enzyme added after about 25 seconds. (G) Same as F, but enzyme added before the bicarbonate.

$\text{HCO}_3^-$  to  $\text{CO}_2$  occurs. Both of these changes are known in themselves to be rather slow, but to be greatly accelerated by the enzyme carbonic anhydrase (7). If, therefore, the catalyzed diffusion theory be true, the overall rate of the entire process should be strongly influenced by this enzyme; according to the purely static theory of Orskov, on the other hand, there is no very obvious reason why such an enzyme should have any effect. The experiments here to be reported, involving in part the addition of more carbonic anhydrase to the hemolytic system and in part the inactivation of that already present in the erythrocytes, have given results which strongly support the theory of catalyzed diffusion.

In Fig. 2 are represented certain volume changes of beef erythrocytes, re-

corded photographically by the method of Parpart (8). The breaks in the curves indicate intervals of 1 second. In Fig. 2 A washed beef cells were placed in  $M/6$   $NH_4Cl$ , in which they swell very slowly. At the point where the sudden rise of the curve begins 0.1 ml. of  $M/2$   $NH_4HCO_3$  was added to 15 ml. of the suspension, the resulting bicarbonate concentration therefore being approximately 0.003  $M$ . The swelling of the cells shown in the photograph passed imperceptibly into hemolysis, which under these conditions required several minutes for its completion, but which with the scale of magnification here used could not be recorded in full on the photographic paper.

The conditions in Fig. 2 B were exactly the same as in Fig. 2 A except that at the beginning of the experiment one drop of a solution containing carbonic anhydrase prepared by the method of Meldrum and Roughton (7) had been added to the cell suspension a few seconds before the beginning of the photographic record. It is obvious that the swelling process was strikingly accelerated; the same was true of the hemolysis which followed. An interesting modification of the experiment is shown in Fig. 2 D in which the enzyme was added after the bicarbonate instead of before it. The almost instantaneous acceleration of the swelling process which it produced is clearly shown. Figs. 2 F and 2 G correspond exactly to Figs. 2 D and 2 B, respectively, except that the concentration of bicarbonate here employed was only half as great, and the rate of swelling both before and after the addition of the enzyme was in consequence considerably slower. The addition of enzyme inactivated by heat had no such effect.

An experiment of the opposite sort is illustrated in Fig. 2 C; in it the enzyme within the cells as well as that added externally in the same amount as in Fig. 2 B had been inactivated before the addition of the bicarbonate by means of sulfanilamide (125 mg. per cent). This substance is known to have a specific inhibitory action on carbonic anhydrase (9). It will be seen that under these conditions the bicarbonate effect almost disappeared. The same general result has been obtained many times under a variety of conditions. It may be noted that the effect of sulfanilamide on the permeability of the erythrocyte to various non-electrolytes such as glycerol or thiourea or even to ammonium salts such as acetate, which do not require an ionic exchange for their penetration (6) is comparatively slight.

While sulfanilamide acts almost instantaneously on the carbonic anhydrase in the external solution, its effect in the interior of the cell is subject to a slight delay. Thus the rate of swelling, on the addition of bicarbonate, of cells suspended in  $NH_4Cl$  saturated with sulfanilamide is distinctly slower after an exposure of 2 minutes than after one of a few seconds; indeed, in the latter case a complete photographic record shows for a short time a gradual decrease in the rate of swelling, after which no further change of rate occurs. Conversely, cells previously saturated with sulfanilamide do not instantly reach their

maximum rate of swelling when placed in a sulfanilamide-free mixture of  $\text{NH}_4\text{Cl}$  and  $\text{NH}_4\text{HCO}_3$ . It may be concluded, therefore, that although in its powers of entering living cells sulfanilamide must be classed as a readily penetrating compound, the erythrocyte is not as permeable to it as it is, for example, to urea, acetamide, various alcohols, etc.

It is also known that carbonic anhydrase can be reversibly inactivated by cyanides (10). The rapidity with which these substances produce an internal effect on the erythrocyte is well shown in Fig. 2 E, in which the addition of 0.05 ml. of 2 M  $\text{KCN}$  to 15 ml. of the stirred cell suspension almost instantly stopped even the maximum rate of swelling attainable under the conditions of the experiment. The inhibitory effect of cyanides on the enzyme present in the interior of the erythrocyte seems to be completely reversible on washing the cells. It should be noted that while cyanides inhibit the catalytic effects of bicarbonates on the entrance of  $\text{NH}_4\text{Cl}$ , they may under appropriate conditions have a catalytic effect of their own, though a considerably weaker one than bicarbonates. This behavior would be expected from certain other similarities between carbonic acid (11) and  $\text{HCN}$  (12) and their salts. An analogous effect of sulfides might also be anticipated (13) and has in fact been indicated by some preliminary experiments (2).

The effects of carbonic anhydrase and of sulfanilamide on the Ørskov effect can also readily be demonstrated and even measured quantitatively with a very satisfactory degree of accuracy by the hemolysis method, which in its simplest form requires no apparatus but a test-tube and a stop-watch. This method, in general, gives results very similar to those already described except that in any given experiment only a single point on the swelling curve instead of the entire curve is open to investigation.

To meet the possible objection that the supposedly pre-hemolytic optical changes recorded in Fig. 2 might be due in part to some undetected non-osmotic type of hemolysis, the experiment was varied so as to avoid hemolysis altogether. In Fig. 3 are shown the volume changes of erythrocytes suspended in isotonic  $\text{NaCl}$  when shrinkage is first produced by the addition of saturated  $\text{NH}_4\text{Cl}$  (0.3 ml. to 15 ml. of suspension) and restoration of the original volume is then accelerated by the further addition, at the point where the slope of the curve changes, of 0.1 ml of  $\text{M}/2$   $\text{NH}_4\text{HCO}_3$ . The rise of the curves somewhat above the starting point in these figures is due to the unavoidable slight dilution of the cell suspension, to which the optical system used for recording is very sensitive. As before, the acceleration of the rate of swelling by bicarbonate (Fig. 3 A), the further acceleration by the addition of enzyme (Fig. 3 B), and the almost complete abolition of the bicarbonate effect by sulfanilamide (Fig. 3 C) are clearly shown.

The effect of sulfanilamide under these various conditions is readily reversible. Even after an exposure of more than 30 hours to a saturated solution



in isotonic NaCl beef erythrocytes recovered their original behavior after being washed twice in a sulfanilamide-free isotonic salt solution. As to the lowest effective concentration of sulfanilamide, the hemolysis method with human erythrocytes under certain conditions (pH 7.6 with a phosphate-phthalate buffer) showed a distinct inhibiting action of as little as 0.03 mg. per cent, which is approximately the same as the minimum concentration ( $2 \times 10^{-6}$  M) found by Mann and Keilin (9). We have some reason to believe, however, that a systematic search for the optimum experimental conditions might enable this value to be carried even lower. As it stands, it is roughly only 1/300 of a

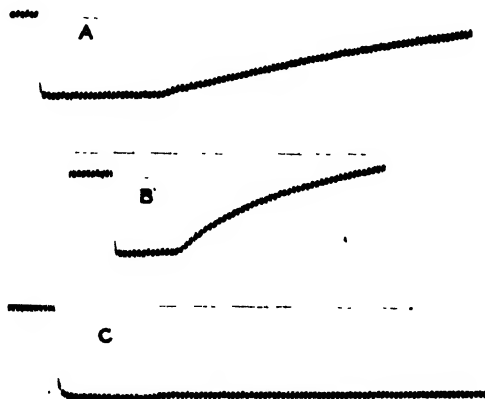


FIG. 3. Volume changes of washed beef erythrocytes originally in  $M/6$  NaCl. 0.3 ml. of saturated  $\text{NH}_4\text{Cl}$  added to 15 ml. of the suspension, followed by 0.05 ml. of  $M/2$   $\text{NH}_4\text{HCO}_3$ .  $22^\circ\text{C}$ . The breaks in the curves indicate intervals of 1 second. (A)  $\text{NH}_4\text{HCO}_3$  alone added. (B) Same as A, but with carbonic anhydrase in the external solution. (C) Same as B in the presence of 125 mg. per cent sulfanilamide.

typical concentration (10 mg. per cent) in the blood of human patients under sulfanilamide therapy. In this connection it may be mentioned that sulfapyridine, as would be anticipated from its behavior with carbonic anhydrase *in vitro* (9), has been found to have no demonstrable inhibitory effect on the volume change of erythrocytes produced by  $\text{NH}_4\text{Cl} - \text{NH}_4\text{HCO}_3$  systems.<sup>1</sup>

### III

As a result of the experiments so far outlined and others of a similar nature it may be said with some confidence that the Ørskov effect is not only influenced by carbonic anhydrase but is largely dependent on it. Support therefore seems to be given to the catalyzed diffusion theory. The principle in question, has,

<sup>1</sup> Sulfathiazole behaves like sulfapyridine.

however, a wider applicability than merely to the behavior of ammonium salts, since it seems to be involved in other more frequently encountered ionic exchanges of a different sort.

Consider, for example, the volume changes of erythrocytes produced by pH changes of the external medium. These cells have long been known to swell in acid and to shrink in alkaline solutions (14-16) at rates which are, in general, rather slow, requiring minutes or possibly sometimes even hours for the attainment of the final equilibrium volume. In the presence of a little bicarbonate, however, we have found that the corresponding times may, under favorable conditions, be shortened to a few seconds; and this fact has since been put to practical use in bringing about rapid equilibration of erythrocytes to solutions of altered pH.

The theory of the effect is very simple (Fig. 1 C). On making an external bicarbonate-free solution more alkaline there is an exchange of  $\text{OH}'$  ions from the solution for  $\text{Cl}'$  ions from the cell. Most of the  $\text{OH}'$  ions combine within the cell to form water, which is osmotically inactive, the  $\text{K}'$  formerly paired with the  $\text{Cl}'$  now being held by the polyvalent hemoglobin ion. The osmotic effect of the  $\text{Cl}'$  is therefore largely lost without being replaced by anything else, and the cell shrinks. The rate of the process, except at high degrees of alkalinity, is quite slow, presumably because of the low concentration of  $\text{OH}'$  ions. If, however, bicarbonate be present (1 D) in a concentration which, though low as compared with that of  $\text{Cl}'$ , is very high as compared with that of  $\text{OH}'$ , a rapid "catalytic" cycle becomes possible between the more alkaline external solution and the more acid cell. On the acid side of the cell membrane the tendency is to form  $\text{CO}_2$  from  $\text{HCO}_3'$ ; on the alkaline side, the reverse. As long as a pH difference exists between the cell and its surroundings and there is  $\text{Cl}'$  to exchange for  $\text{HCO}_3'$  the cycle can continue (see in this connection 17). The exchange of  $\text{Cl}'$  for  $\text{HCO}_3'$  is known to be an extremely rapid process (18, 19), and in the presence of carbonic anhydrase the other parts of the cycle can also become rapid. The final equilibrium should therefore be approached far more quickly in the presence than in the absence of bicarbonate.

It is easy to show the effect of bicarbonates and of carbonic anhydrase on the rates of shrinkage and swelling produced by pH changes. In Fig. 4 A beef erythrocytes were suspended in a buffered salt solution at pH 7.01. Enough  $\text{NaOH}$  was then added to change the pH to 8.35. The shrinkage that followed was so slow that no attempt was made to follow it photographically to its end. Instead, after about 90 seconds enough acid was added approximately to restore the original pH; and a slow return towards the original volume occurred. It will be seen in Fig. 4 B that the presence of a little bicarbonate greatly accelerated both processes, particularly the swelling. The addition of carbonic anhydrase (Fig. 4 C) still further increased the rapidity of shrinkage, though under the conditions of this particular experiment it did

not make very much difference in the swelling process. In the presence of sulfanilamide, on the other hand (Fig. 4 D), the same amounts of bicarbonate and of enzyme as in Fig. 4 C were almost without effect.

Particularly striking results can be obtained with cells suspended in isotonic sugar solutions made alkaline with NaOH. Under these conditions shrinkage

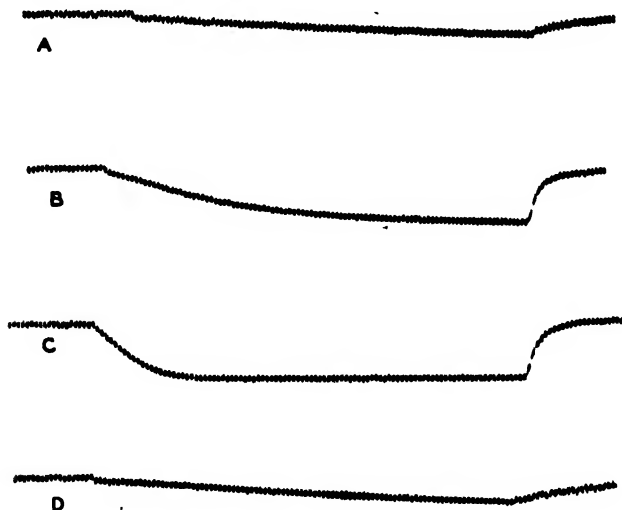


FIG. 4. Volume changes of washed beef erythrocytes originally in  $M/6$  NaCl buffered at pH 7.01 with a phosphate-phthalate mixture.  $22^{\circ}\text{C}$ . The breaks in the curves indicate intervals of 1 second. (A) Sufficient NaOH added to change the pH to 8.35, then sufficient HCl approximately to restore its original value. (B) Same as A in the presence of  $0.002\text{ }M$   $\text{NaHCO}_3$ . (C) Same as B with carbonic anhydrase in the external solution. (D) Same as C in the presence of 125 mg. per cent of sulfanilamide.

occurs fairly rapidly, but can be greatly accelerated by bicarbonates. Fig. 5 A represents the behavior of a stirred suspension of beef erythrocytes in a  $0.3\text{ }M$  sucrose solution which, at the point where the descent of the curve begins, was suddenly brought to pH 8.9 with NaOH. The resulting shrinkage of the cells was still in progress, though nearly complete, in 90 seconds. Fig. 5 B represents a similar experiment, begun in exactly the same way, but with the addition after 15 seconds of a small amount of bicarbonate ( $0.002\text{ }M$ ). The response of the cells to the presence of the bicarbonate is strikingly shown. A second addition of the same amount of bicarbonate after the descent of the

curve had been completed (6 seconds from the end of the record) can be seen to have had almost no further effect. The pH of the solution in this case was little changed by either addition of bicarbonate, the chief effect of the latter being greatly to hasten the attainment of an otherwise predetermined equilibrium—which is the essence of the behavior of a catalyst.

Another series of experiments, not here represented by photographs illustrate another aspect of the "catalytic" effect of bicarbonates. In each case

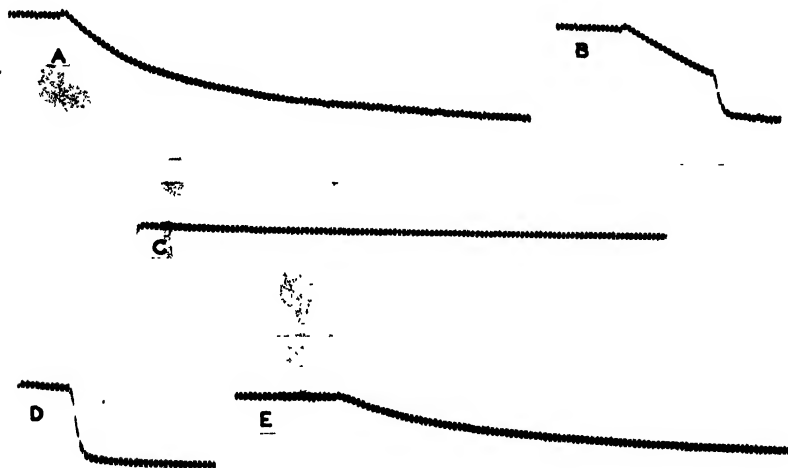


FIG. 5. Volume changes of washed beef erythrocytes originally in 0.3 M sucrose. 22°C. The breaks in the curves indicate intervals of 1 second. (A) Sufficient NaOH added at the descent of the curve to bring the pH to 8.9. (B) Same, but at the point of the second, more rapid descent  $\text{NaHCO}_3$  (0.002 M) was added. An equal amount of  $\text{NaHCO}_3$  was again added 6 seconds from the end of the record. (C) Cells in 0.3 M sucrose. (D) Same as C but at the point of the rapid descent  $\text{NaHCO}_3$  (0.002 M) was added. (E) Same as D in the presence of 125 mg. per cent of sulfanilamide.

the cells were placed in a 0.3 M sucrose solution made alkaline with NaOH, and bicarbonate was added as before, but after different times in the different experiments. (See also in this connection Fig. 6.) The rapid shrinkage induced by this addition was very different in magnitude according to the time at which it occurred, but in each case was approximately that required to complete the process of shrinkage already in progress. As before, a further addition of bicarbonate after the attainment of the final cell volumes had only negligible effects.

Bicarbonate effects of this type, like those involving ammonium salts, are greatly reduced by inhibitors of carbonic anhydrase such as cyanides and sulf-

anilamide, as is shown in the series of experiments represented in Fig. 5. In Fig. 5 C washed beef erythrocytes were suspended in 0.3 M sucrose, in which a very slow shrinkage takes place, presumably in consequence of the exchange of  $\text{Cl}'$  ions from the cell for  $\text{OH}'$  ions from the surrounding non-electrolyte solution (for a discussion of this behavior, see Jacobs and Parpart, 20, 21, and Wilbrandt, 22). The addition of bicarbonate to such a suspension has the double effect of greatly increasing the concentration of  $\text{OH}'$  and at the same

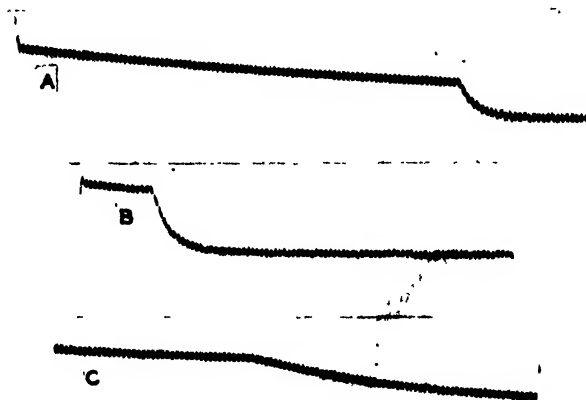


FIG. 6. Volume changes of washed beef erythrocytes in  $\text{m}/12 \text{ Na}_2\text{SO}_4$  buffered at pH 7.4. (The slight hypotonicity of the solution was intentional to avoid the possibility of direct osmotic effects during the course of the experiment in the same direction as those caused by the ionic exchange.)  $22^\circ\text{C}$ . The breaks in the curves indicate intervals of 1 second. (A) At the point of sudden descent of the curve  $\text{NaHCO}_3$  (0.002 M) was added. A second equal amount added a few seconds from the end of the curve had a negligible effect. (B) Same as A, but the first addition of bicarbonate was made earlier. (C) Same as B in the presence of 125 mg. per cent of sulfanilamide.

time bringing into action the catalytic mechanism already discussed. The resulting shrinkage (Fig. 5 D) was the most rapid that has as yet been obtained by any method involving the effects of increased alkalinity, being virtually complete in 4 or 5 seconds. The effect of sulfanilamide, shown in Fig. 5 E, is greatly to retard this change, which in its presence was not finished even at the end of 90 seconds. It should, of course, be noted in this, as in other cases that bicarbonates are not completely ineffective in the absence of active carbonic anhydrase, since the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$  can take place in either direction without the assistance of any enzyme, though at a greatly reduced rate.

The general conclusion to be drawn from the experiments discussed in the present section, and from other similar ones that have not been mentioned, is that the "catalytic" effect of bicarbonates on the alterations of the cell volume induced by pH changes is as striking as that previously described for ammonium salts. Like the latter, it is dependent for its full effectiveness on the presence of the enzyme carbonic anhydrase.

#### IV

In the cases so far discussed the rôle of  $\text{CO}_2$  and  $\text{HCO}_3'$  in promoting ionic exchanges is fairly obvious. A somewhat more surprising instance of an apparent catalytic effect of bicarbonates has been found in connection with the volume changes of erythrocytes associated with the exchange of  $\text{Cl}'$  from the cells for  $\text{SO}_4''$  from the surrounding solution. This phenomenon, which depends on the fact that one bivalent ion is osmotically less effective than the two univalent ions for which it must be exchanged, has recently been studied by Parpart (23), who worked exclusively with well washed erythrocytes from which most of the bicarbonate had presumably been removed. Under these conditions he found that the rate of exchange of the two ions is rather slow, the times for one-half completion of the process at  $20^\circ\text{C}$ . frequently being measured in minutes rather than in seconds.

In repeating some of Parpart's experiments with  $M/9$  and  $M/12$  solutions of  $\text{Na}_2\text{SO}_4$  buffered at different pH values by a phosphate-phthalate mixture, we were surprised to find that the addition of low concentrations of sodium bicarbonate rapidly brought about the attainment of the final volume—frequently within a few seconds. That the effect was not merely that of alkalinity *per se*, which Parpart had studied, was shown by the fact that the pH changes of the buffered solution produced by low concentrations of bicarbonate were usually rather slight. Furthermore, even with the most careful pH control the bicarbonate was found to have a relatively enormous effect of its own, which in several important respects resembled that described in the preceding sections.

For example, at a constant pH value the cell volume finally attained in a buffered solution of  $\text{Na}_2\text{SO}_4$  was, within reasonable limits, independent of the amount of bicarbonate added, though the rates of its attainment varied as before. Furthermore, at constant pH, the addition of more bicarbonate after the equilibrium volume had been reached had little effect. Likewise, the magnitude of the acceleration following the addition of the bicarbonate depended on the time of its addition. Finally, and perhaps most important, the bicarbonate effect largely disappeared in the presence of sulfanilamide or KCN, indicating that, as before, the enzyme carbonic anhydrase was playing a part in the process. Some of these peculiarities of behavior are shown in Fig. 6.

Though the exact mechanism of action of  $\text{HCO}_3'$  on the  $\text{Cl}'\text{-SO}_4''$  exchange is still under investigation, a tentative explanation of it may be suggested. As has already been noted above, the exchange of  $\text{HCO}_3'$  for  $\text{Cl}'$  is known to be extremely rapid. When, therefore, sodium bicarbonate is added to a solution of a salt with a much more slowly penetrating anion such as  $\text{SO}_4''$  there will be a tendency, regardless of this ion, for an immediate approach toward the Donnan distribution  $[\text{Cl}']_i/[\text{Cl}']_o = [\text{HCO}_3']_i/[\text{HCO}_3']_o$ , the subscripts  $i$  and  $o$  indicating inside and outside respectively. It is easy to show that for a ratio of volume of solution to volume of cells of 600:1, which was approximately that involved in the present experiments, an exchange of equal numbers of the two ions in question would lead to a very high value of  $[\text{HCO}_3']_i$  as compared with  $[\text{HCO}_3']_o$ , *i.e.*; to a considerably increased internal alkalinity of the cell. This, by the mechanism already discussed, would lead to a rapid shrinkage of the cell; and it is reasonable to suppose that the immediate volume changes on the addition of bicarbonate recorded in Fig. 6 may be largely due to this factor. Indeed, a situation in which it is the only factor of significance is provided by the use of isotonic sodium citrate instead of sodium sulfate for the external solution. Since erythrocytes are almost impermeable to the citrate ion (2, 24) their behavior in a solution of sodium citrate, both before and after the addition of bicarbonate, in certain respects should be, and has actually been found to be, essentially the same as that in a solution of sucrose, which has already been discussed.

The subsequent exchange of  $\text{SO}_4''$  for  $\text{HCO}_3'$  cannot be followed by the method of volume changes, since theoretically about the same shrinkage should result either from the exchange of a given number of  $\text{Cl}'$  ions from the cell for an equal number of  $\text{HCO}_3'$  ions, or for half the number of  $\text{SO}_4''$  ions, from the surrounding solution. It has, in fact, been found experimentally that cells first equilibrated with  $m/6$   $\text{NaHCO}_3$  and then placed in  $m/9$   $\text{Na}_2\text{SO}_4$  show an almost complete absence of the moderately slow volume changes that occur under these conditions with cells previously equilibrated with  $m/6$   $\text{NaCl}$ .

While the most obvious part of the volume change produced by the addition of bicarbonate to a  $\text{Na}_2\text{SO}_4$  solution therefore appears to be associated with a temporary tendency towards increased intracellular alkalinity (Fig. 1 F), it would seem theoretically that under the conditions of these experiments  $\text{SO}_4''$  ought to enter the cell more rapidly after most of the  $\text{Cl}'$  of the cell has been replaced by  $\text{HCO}_3'$  than before, in consequence of the operation of the cycle represented in Fig. 1 G. Some experimental evidence in favor of this view has been obtained by studying the increases in cell volume on the addition of small amounts of  $\text{NaCl}$  to the external solution at different times during and after the shrinkage process; but more work along these lines will be required before this evidence can be considered complete. There is also a

possibility, suggested by the work of Parpart, that the increased intracellular alkalinity resulting from the exchange of  $\text{Cl}'$  for  $\text{HCO}_3'$  may make the cell more permeable to the  $\text{SO}_4''$  ion. At all events, there is no doubt that the otherwise rather slow shrinkage of erythrocytes in buffered  $\text{Na}_2\text{SO}_4$  solutions can be greatly accelerated by the presence of low concentrations of bicarbonates, this effect being, in turn, strongly influenced by the enzyme carbonic anhydrase.

What is true of the erythrocyte is doubtless also true of other cells which are permeable to anions. The presence of carbonic anhydrase in large quantities in tissues such as the gastric mucosa (25), the gills of fishes (26), etc., in which there is reason to believe that exchanges of anions of different sorts takes place, is of possible interest in this connection. In conclusion, it may be noted that in the case of cells which are more permeable to cations than to anions, of which many examples are known in both animals and plants, it would be reasonable to expect that ammonia might perhaps take part in a "catalytic" cycle similar to that here suggested for carbonic acid in the case of anion-permeable cells (27). This field has, however, not yet been investigated.

#### SUMMARY

1. The acceleration by bicarbonates of the swelling and hemolysis of erythrocytes in solutions of ammonium salts, first reported by Ørskov, is strikingly dependent upon carbonic anhydrase, being almost abolished by inhibitors of this enzyme such as KCN and sulfanilamide, and under suitable conditions being enhanced by its addition to the external solution. This behavior gives support to the theory of "catalyzed diffusion" as an explanation of the Ørskov effect.

2. The inhibitory effects of both sulfanilamide and KCN seem to be capable of complete reversal on washing the erythrocytes in isotonic salt solutions. The full effect of KCN appears almost instantly; that of sulfanilamide requires a period measured in seconds, or possibly even in minutes, to reach its maximum, the delay presumably being due to the slower penetration of the erythrocyte by this substance. Under favorable conditions the effect of concentrations of sulfanilamide of a few hundredths of a milligram per cent can be demonstrated. No similar effects have been obtained with sulfapyridine.

3. Bicarbonates also have a "catalytic" effect on the response of the internal pH of erythrocytes to changes in that of their surroundings. The resulting volume changes of the cell, which otherwise frequently require many minutes for their completion, may take place within a few seconds in the presence of low concentrations of bicarbonates. At a given pH value the effect of the latter substances is chiefly on the rate of the change and only to a minor extent on its magnitude. It may be further accelerated under appropriate conditions



by the addition to the cell suspension of carbonic anhydrase, and can be almost abolished by KCN and by sulfanilamide.

4. Volume changes of erythrocytes associated with exchanges of  $\text{Cl}'$  for  $\text{SO}_4''$  ions are greatly accelerated by low concentrations of bicarbonates, this effect being likewise dependent upon carbonic anhydrase. There is some evidence that in this case the exchange takes place, at least in part, in two steps:  $\text{Cl}'$  for  $\text{HCO}_3'$  and  $\text{HCO}_3'$  for  $\text{SO}_4''$ .

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# VISUAL ACUITY AND ILLUMINATION IN DIFFERENT SPECTRAL REGIONS

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## I

### INTRODUCTION AND PROCEDURE

It has long been known that visual acuity increases with the illumination (Uthoff, 1886, Koenig, 1897). A precise study of this function with white light (Shlaer, 1937) has shown that the data fall into two distinct functions, one at intensities below about 0.1 photon, and the other at intensities above that. This separation is understandable in terms of the duplicity theory.

Because of the different sensibilities of the rods and cones in the spectrum, it is to be expected that the short wave end of the spectrum will give the greatest separation between the rod and cone functions, that the long wave end will give practically a pure cone function, and that the intermediate spectral regions will give intermediate separations. Such a situation has already been found in intensity discrimination, flicker, dark adaptation, and is indicated in Koenig's (1897) visual acuity data (Hecht, 1937). We therefore determined to measure the relation between visual acuity and illumination in lights of different restricted spectral regions, using the same two test objects as in the previous study with white light. These were a broken circle (C) with the break equal to the width of the line forming the circle and one-fifth its outside diameter, and a grating having opaque and transparent bars of equal size. Because visual acuity measurements are extremely difficult and tedious to make, and because the intermediate spectral regions give results of but secondary importance, most of the measurements to be presented here were made with only the two extremes of the visible spectrum, the red and the blue.

The apparatus we used has already been described in detail (Shlaer, 1937). It consists essentially of a device in which the test object is presented at a fixed distance of 1 meter from the eye in the center of a uniform field about 30° in diameter, and is viewed through an artificial pupil. The size of the test object may be changed continuously over a 1 to 100 range by means of a variable focus projection system, while the illumination is varied in small steps by means of neutral filters. The neutral filters were calibrated in light transmitted by the color filters, using a Koenig Martens photometer. The heterochromatic den-

sity of the color filters was determined by comparison with similar filters calibrated by Hecht and Smith and used in the flicker investigation by Hecht and Shlaer (1936).

The procedure was for the most part as already described. The subject was first completely dark adapted. His visual acuity was then determined at the lowest intensity possible after he had become completely adapted to that field brightness. The intensity was then raised a suitable amount and the measurement repeated after the subject had again become adapted to the new brightness. This was continued till a little more than half the complete intensity range had been covered. The very next day measurements were begun a little below the highest point reached in the previous session and carried to the end of the intensity range. In this way exceptional fatigue of the observer was avoided, while the overlapping points of the two consecutive sessions gave assurance that no change in the sensitivity of the observer had occurred. Other differences from the previously described procedure will be noted below in the relevant sections.

Measurements were made with the right eye of E.L.S. and of A.M.C., while S.S. was the manipulator. E.L.S. and A.M.C. had served as observers in the previous study with white light.

## II

### *Results with the C Test Object*

Measurements with the C as test object were made with light of two different spectral regions: (1) that transmitted by Eastman Kodak Wratten filter No. 70 (the color is red of dominant wavelength  $670\text{ m}\mu$ ), and (2) that transmitted by Wratten filter No. 75 and Corning filter No. 428 (the color is blue of dominant wavelength  $490\text{ m}\mu$ ). Filter 75 was chosen in preference to 76 (violet, of dominant wavelength  $450\text{ m}\mu$ ) because its visual density is much lower, enabling maximal visual acuities to be attained, while the displacement of the rod and cone sections of the data is nearly the same as that produced by violet light. A fixed pupil of 2 mm. diameter was used for both.

The Corning filter served two purposes. It absorbed the small amount of red light transmitted by the blue Wratten filter, and was ground to be a lens of  $-1$  diopter (focal length of 1 meter). The latter overcomes the difficulty of the emmetropic eye in focusing the test object in blue light because of chromatic aberration.<sup>1</sup>

<sup>1</sup>This phenomenon may be simply demonstrated by means of the "monochromat" series of Wratten filters of Eastman Kodak Company. If one looks through these filters at some distant object, one finds that fine detail is readily perceived through all but filters 75 (blue) and 76 (violet). With these a lens of about  $-1$  diopter at once restores the sharpness of vision.

The data are given in Table I. Each datum is the average of determinations made in two different complete runs. Those in black type represent measure-

TABLE I  
*Visual Acuity and Illumination, C Test Object*

The bold faced numbers represent determinations with peripheral vision in a subjectively colorless field; the italic numbers, with parafoveal vision in a colored field; and the rest, with foveal vision in a colored field.

Red ( $\lambda = 670 \text{ m}\mu$ )			Blue ( $\lambda = 490 \text{ m}\mu$ )		
Log <i>I</i> in photons	Log visual acuity		Log <i>I</i> in photons	Log visual acuity	
	A.M.C.	E.L.S.		A.M.C.	E.L.S.
1	2	3	4	5	6
-1.875	-1.434	-1.481	-3.865	<b>-1.468</b>	
-1.546	-1.192	-1.304	-3.392	<b>-1.181</b>	<b>-1.363</b>
-1.243	-0.954	-1.055	-3.084	<b>-1.067</b>	<b>-1.233</b>
-0.841	-0.657	-0.715	-2.785	<b>-0.949</b>	<b>-1.108</b>
-0.522	-0.435	-0.468	-2.269	<b>-0.824</b>	<b>-0.885</b>
-0.219	-0.269	-0.314	-1.961	<b>-0.782</b>	<b>-0.778</b>
0.193	-0.104	-0.121	-1.662	<b>-0.734</b>	<b>-0.708</b>
0.512	0.018	0.016	-1.317	<i>-0.649</i>	<b>-0.654</b>
0.815	0.118	0.108	-1.009	<i>-0.564</i>	<b>-0.563</b>
1.147	0.213	0.199	-1.009		<b>-0.653</b>
1.466	0.258	0.251	-0.710	<i>-0.482</i>	<b>-0.446</b>
2.086	0.302	0.324	-0.710	-0.720	-0.520
2.718	0.328	0.353	-0.273	<i>-0.377</i>	<b>-0.270</b>
3.439	0.364	0.370	-0.273	-0.460	-0.310
4.154	0.374	0.387	0.035	<i>-0.278</i>	<b>-0.126</b>
4.776	0.381	0.400	0.035	-0.316	-0.148
			0.334	<b>-0.140</b>	<b>-0.002</b>
			0.759	0.019	0.144
			1.068	0.118	0.215
			1.367	0.212	0.267
			1.883		0.321
			2.191	0.302	
			2.490		0.389
			2.835	0.347	
			3.143		0.403
			3.442	0.371	
			3.879		0.410
			4.187	0.389	
			4.486	0.402	0.411

ments in subjectively colorless fields. The data of columns 2 and 5 are shown in Fig. 1 and those of columns 3 and 6 in Fig. 2. To separate the measurements, the data for red light are plotted 0.5 log units lower on the log visual acuity axis.

It can be seen from these figures that the data with blue light fall into two distinct curves, one (for the rods) at low intensities of illumination and one (for the cones) at the higher intensities. The data with red light fall into a single continuous curve with the possible exception of the first point at the very lowest intensity.

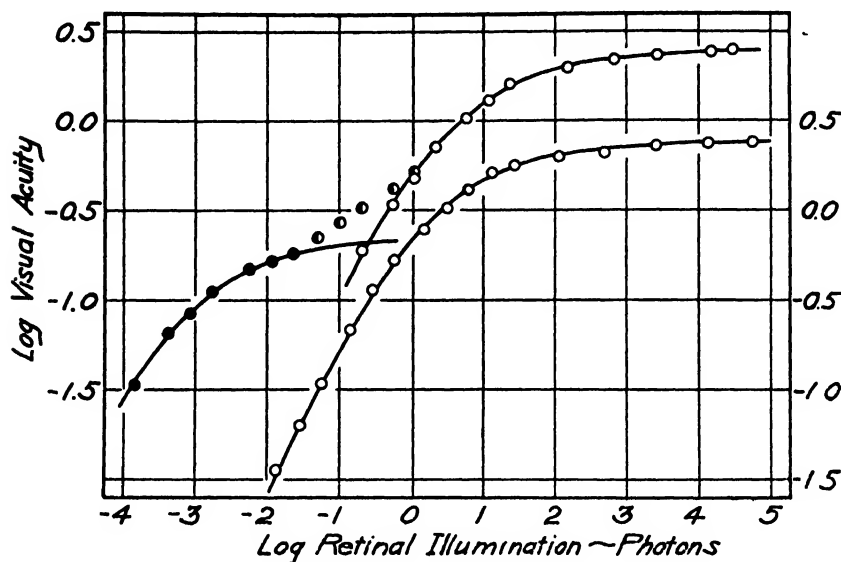


FIG. 1. The data of A. M. C. with the C test object from Table I, columns 2 and 5. The lower data (ordinates at the right) are with red light. The upper data (ordinates at the left) are with blue light. The filled circles are the measurements made with the periphery in a subjectively colorless field and represent pure rod function. The half-filled circles are the measurements made with the parafovea in a subjectively colored field and represent combined rod and cone function. All other circles are measurements made with the fovea and represent pure cone function. The curves drawn through both the rod and cone data are of equation (1) where  $m = n = 2$  and visual acuity is proportional to  $x^n$ .

The curves drawn through all the cone data are theoretical and are the same ones previously drawn through the cone data with white light. These curves are graphs of Hecht's (1937) stationary state equation for the photoreceptor process:

$$KI = x^n / (a - x)^m \quad (1)$$

where  $I$  is the intensity of illumination,  $(a - x)$  is the concentration of photo-products,  $m$  and  $n$  are the orders of the photochemical and thermal reactions respectively, and  $K$  is a constant. In the form used here, visual acuity is proportional to  $x^n$  while  $m$  and  $n$  are both 2. Because the rod data obtained with

blue light are more extensive, a more critical selection of the theoretical curve is possible. In these figures the curves drawn through the rod data are the same ones drawn through all the cone data.

Comparison with Fig. 1 of the earlier work with white light (Shlaer, 1937) shows that the rod function in blue light is situated about 1.5 log units lower on the intensity axis than in white light. This is in accordance with expectation and is similar to the behavior of other visual functions (see review by Hecht, 1937). The intensity scale is, by definition, that of the cones. Because the spectral sensibility of the rods lies further towards the blue end of the spectrum

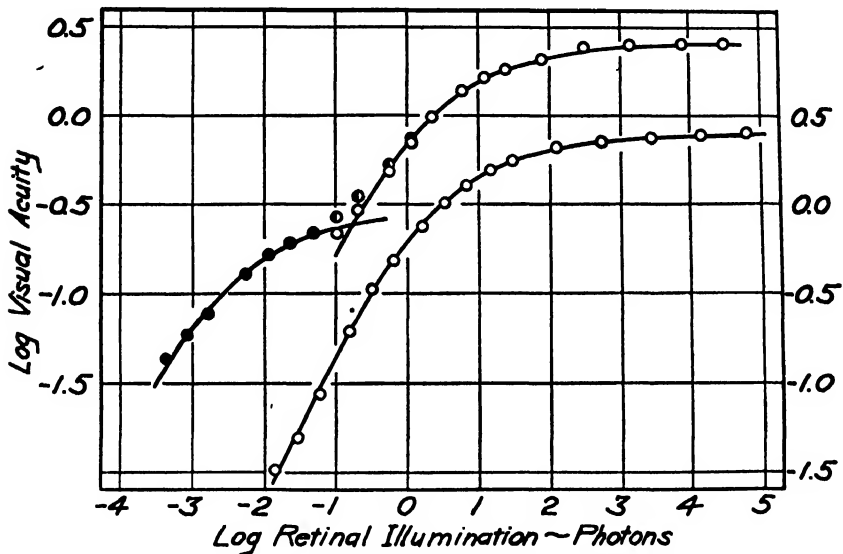


FIG. 2. The data of E.L.S. with the C test object from Table I, columns 3 and 6. The symbols and curves are as in Fig. 1.

than does that of the cones, a blue light of a given cone intensity will have a greater brightness to the rods than a white light having the same cone intensity. This is the familiar Purkinje phenomenon. Therefore any visual function of the rods dependent on intensity will be shifted to lower intensities on a cone brightness scale when the short wave end of the spectrum is used.

With white light there exists an intensity range at the beginning of the cone function where foveal fixation of the test object gives lower visual acuities than parafoveal fixation (Shlaer, 1937). This was ascribed to the behavior of parafoveal cones.

We have now studied this phenomenon somewhat more fully by determining at each intensity of this region the visual acuity first with the more sensitive parafoveal region, and then with foveal fixation. The former values appear in italics in the table and in half-filled circles in the figures.

It will be seen that this phenomenon is present in the measurements made with blue light, but is entirely absent in those made with red light. This must mean that those values of visual acuity that lie above the curves at the transition region are mediated by both rods and cones. For if they were due only to the parafoveal cones, they should appear also in the red light data; moreover, if they were due only to the rods, then these points should appear at the same intensity above the rod threshold in both the white and blue light data. This demands that these points lie about 1.5 log units lower on the intensity axis (cone scale) since that is the amount that the rod curve has been shifted in blue light as compared to its position in white light. Neither of the above two conditions obtain in these data; on the contrary, these points appear only at the region of transition from rod to cone function. It is therefore clear that these points are the result of the cooperative activity of both the rods which are near their maximal activity and the cones whose activity is just becoming apparent.

It has long been known that the brightness of a field may be the result of simultaneous activity of both rods and cones. Thus the luminosity function determined at certain intermediate intensities is intermediate between the bright (cone) function and the dim (rod) function (Koenig, 1891). However, this is the first time, we believe, that an increase in a visual function dependent on brightness has been demonstrated to take place when both rods and cones act simultaneously.

### III

#### *Results with the Grating Test Object*

The measurements with the grating in colored light were made under somewhat different conditions than in white light. Because the colored filters reduce the illumination by a factor of 100 or even 1000, the ground glass sources used with white light were removed and the apparatus was used as for the C so that sufficient brightness for maximum visual acuities was available. The small central dot used previously as a plane-finding fixation mark served adequately at high intensities but was quite invisible at low ones. To overcome this deficiency a ring of 62 mm. outside diameter and 7.5 mm. wall thickness was mounted on the field lens. Such a ring is visible at the lowest intensities used.

It was shown (Shlaer, 1937) that for grating resolution a pupil less than 2.3 mm. in diameter is the limiting factor at maximal brightness in white light. This limitation is the result of two factors. (1) A repetitive pattern such as the grating gives rise to diffracted spectra in the plane of the pupil. For every point in the source there is formed an undeviated and undiffracted image (zero order spectrum) to both sides of which lie the different higher order spectra in which the deviation is proportional to the wavelength. (2) For a lens to form a true image of such a repetitive object it must be large enough to transmit the entire diffraction pattern formed by it (Abbe, 1873). As the lens aperture is made smaller and smaller the image becomes poorer and poorer until, when only

the zero order spectrum alone is transmitted, no discernible pattern at all can be detected in the image plane. For the human eye in white light the pupil need be only large enough to transmit the zero order spectrum and most of one first order spectrum in order that the retina be able to resolve the image. Since the red end of a diffraction spectrum is furthest from the undiffracted source image,

TABLE II  
*Visual Acuity and Illumination, Grating Test Object*

The bold faced numbers represent determinations made in a subjectively colorless field.

Red ( $\lambda = 670 \text{ m}\mu$ ) 2 mm. pupil			Red ( $\lambda = 670 \text{ m}\mu$ ) 3 mm. pupil			Blue ( $\lambda = 490 \text{ m}\mu$ ) 2 mm. pupil		
Log $I$ in photons	Log visual acuity		Log $I^*$ in photons	Log visual acuity		Log $I$ in photons	Log visual acuity	
	A.M.C.	E.L.S.		A.M.C.	E.L.S.		A.M.C.	E.L.S.
1	2	3	4	5	6	7	8	9
-1.546		-0.818	-1.210	-0.440	-0.650	-3.392	<b>-0.982</b>	<b>-1.007</b>
-1.243	-0.566	-0.652	-0.907	-0.313	-0.501	-3.084	<b>-0.906</b>	<b>-0.900</b>
-0.841	-0.372	-0.383	-0.505	-0.154	-0.377	-2.786	<b>-0.832</b>	<b>-0.836</b>
-0.522	-0.226	-0.193	-0.186	-0.073	-0.225	-2.269	<b>-0.764</b>	<b>-0.738</b>
-0.219	-0.106	-0.118	0.117	0.015	-0.138	-1.961	<b>-0.665</b>	<b>-0.649</b>
0.193	-0.012	-0.025	0.529	0.092	-0.017	-1.662	<b>-0.615</b>	<b>-0.610</b>
0.512			0.848	0.129	0.046	-1.317	-0.517	-0.476
0.815	0.075	0.081	1.151	0.172	0.111	-1.009	-0.401	-0.336
1.466	0.137	0.140	1.802	0.215	0.197	-0.710	-0.314	-0.223
2.086	0.162	0.167	2.422	0.263	0.254	-0.273	-0.185	-0.131
2.718	0.179	0.195	3.054	0.300	0.308	0.035	-0.101	-0.064
3.439	0.192	0.209	3.775	0.320	0.318	0.334	-0.017	-0.009
4.154	0.200					0.759	0.077	0.076
						1.367	0.145	0.151
						2.191	0.192	0.206
						2.835	0.234	0.254
						3.442	0.262	0.286
						4.187	0.261	0.289

\*The values of log  $I$  in this column are those of column 4 to which the value 0.336 has been added. The added value represents the logarithm of the ratio of the effective pupil areas; *i. e.*, the actual areas corrected for effectiveness from the data of Stiles and Crawford (1933). The difference between the effective and actual area ratios is small, the logarithm of the latter being 0.352.

it follows that a larger pupil is necessary to achieve maximum visual acuity in red light than in any other colored or white light. It will be shown below that a diameter of 3 mm. is more than adequate to eliminate the pupil as a limiting factor even for red light. For this reason the relation of visual acuity and illumination was determined with both a 2 and 3 mm. pupil in red light and with only a 2 mm. pupil in blue light.

The data are given in Table II. Each value of log visual acuity represents



the average of determinations obtained in three complete runs with the exception of the initial value of column 3 which is the average of only two runs. As with the white light data, it is possible to plot the observations of both observers on the same grid and to fit them with one curve. The only exception is the data of column 6. These data were taken during an interval shortly after E.L.S. recovered from a severe cold. The result is that the entire curve is shifted about 0.5 log units higher on the intensity axis as compared to his previous and also subsequent data, as well as compared to the data of A.M.C. in

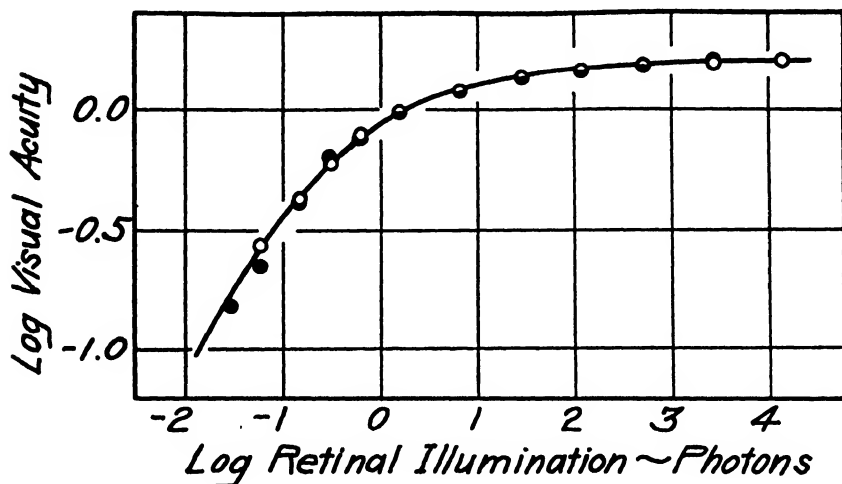


FIG. 3. The data of both observers with the grating test object, red light, and a pupil of 2 mm. diameter. The unfilled circles are for A.M.C. from Table II, column 2. The filled circles are for E.L.S. from Table II, column 3. The curve drawn through these data is of equation (1) where  $m = n = 2$  and visual acuity is proportional to  $x^n$ .

column 5. For this reason all of these data are plotted 0.5 log units lower on the intensity axis than given by the table.

The data with red light and a 2 mm. pupil are plotted in Fig. 3; those with red light and a 3 mm. pupil in Fig. 4; and those with blue light in Fig. 5. The curve drawn through the data of Fig. 3 is theoretical and is the same one drawn through the cone data with white light; that drawn through the data of Fig. 4 and through the cone data of Fig. 5 is of the same equation but of half the slope as that in Fig. 3. The rod data of Fig. 5 appear about one log unit lower on the intensity axis as compared to those of white light, but are still too scanty to permit of any critical curve fitting.

It will be recalled that the white light grating data were adequately described by the same equation that described the C data, namely,

$$KI = x^n / (a - x)^m \quad (1)$$

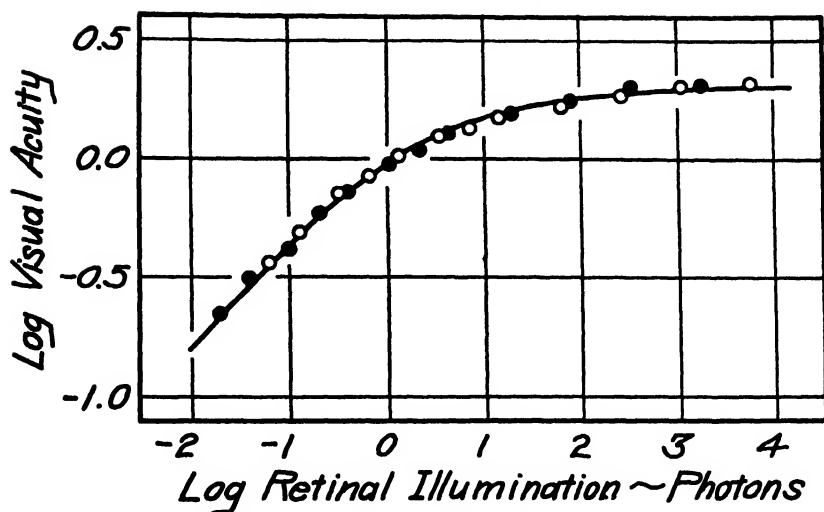


FIG. 4. The data of both observers with the grating test object, red light, and a pupil of 3 mm. diameter. The unfilled circles are for A.M.C. from Table II, column 5. The filled circles are for E.L.S. from Table II, column 6, but plotted 0.5 log units lower on the intensity axis than given in the table (see text). The curve drawn through the data is of equation (1) where  $m = n = 2$  and visual acuity is proportional to  $x$ .

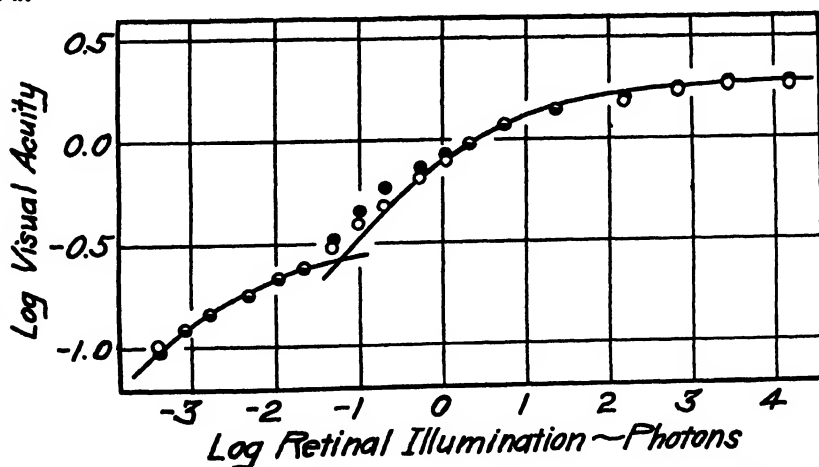


FIG. 5. The data of both observers with the grating test object, blue light, and a pupil of 2 mm. diameter. The unfilled circles are for A.M.C. from Table II, column 8. The filled circles are for E.L.S. from Table II, column 9. All the data below a  $\log I$  value of  $-1.5$  were made in a subjectively colorless field and represent pure rod function; those made at higher intensities were made in a colored field. The curves drawn through both sections of the data are of equation (1) where  $m = n = 2$  and visual acuity is proportional to  $x$ .

where both  $m$  and  $n$  are 2, and visual acuity is proportional to  $x^n$ . These data were taken under conditions where the pupil size was the limiting factor for visual acuity. The data in Fig. 3, where the 2 mm. pupil lowers the maximum visual acuity even more because of the red light, are also adequately described by the same equation. However, when the pupil diameter is eliminated as a limiting factor, as in the data of Fig. 4, this form of the equation is quite inadequate. The best fit to these data was given by the form of this equation where both  $m$  and  $n$  are again 2 but where visual acuity is proportional to  $x$  instead of to  $x^n$ . Since  $n$  is 2, it means that the latter curve has half the slope of the former.

It is difficult to believe that the difference in pupil size can have such a profound effect on the entire course of this relationship. It may be that the small pupil affects only the upper part of the data where the visual acuity values approach the limit set by it. Perhaps the explanation of this apparent effect lies along the following lines. The graphs of the various forms of equation (1) may be arbitrarily divided for convenience into an initial rising straight line portion, and an upper turning-off to an asymptote. Since the visual acuity range of the grating data is quite small, the first characteristic is a relatively poorer criterion than the second which then is practically the dominant one in curve fitting. If the limiting pupil affects only the higher visual acuity values, the data would turn off to an asymptote more sharply, giving a good fit with the steeper curve. This is clearly shown in Fig. 6 where the data for A.M.C. with the two different pupils in red light are plotted together, those with the 2 mm. pupil being moved slightly to the right on the intensity axis to bring the lower parts of the two curves into superposition.

Visual acuity is, by definition, a measure of angular separation. Essentially, a linear distance is measured. The two test objects used in this investigation differ with respect to the resolvable areas involved. In the broken circle the area is a function of the square of the separation since both dimensions of the gap change simultaneously, while in the grating the area is a linear function of the separation since the length remains constant. If area is the important variable in detail perception, then the relation of visual acuity (a distance measure) to illumination should be twice as steep for the grating as for the broken circle. The previous data with white light (Shlaer, 1937) seemed to show that this relationship of visual acuity and illumination was the same for both test objects. The conclusion was then drawn that detail perception was a function of linear distance rather than of area. However, if the foregoing interpretation of the red light data is correct and it is accepted that the relation of visual acuity to illumination is half as steep for the grating as for the broken circle, then the previously drawn conclusion must be invalid.

The curve drawn through the blue cone data in Fig. 5 is the same as the one drawn through the red 3 mm. pupil data in Fig. 4. The first three points of the

colored field data in Fig. 5 lie above the theoretical cone curve. This may, perhaps, mean that here, as with the C, there is a cooperation between rods and cones to produce a higher visual function than does either alone, the one log unit shift in the rod curve produced by blue light being essential to bring this out.

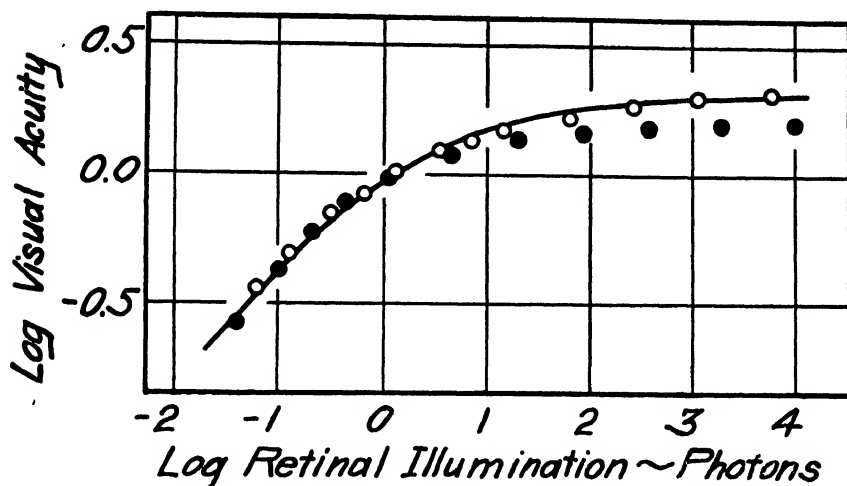


FIG. 6. The data of A.M.C. with the grating test object and red light, showing the effect of a limiting pupil upon the relation between visual acuity and illumination. The filled circles are from Table II, column 2, where the 2 mm. pupil limits the maximum visual acuity attainable. The unfilled circles are from Table II, column 5, where the 3 mm. pupil is no longer limiting. The curve drawn in this figure is of equation (1) where  $m = n = 2$  and visual acuity is proportional to  $x$ .

#### IV

#### *Factors Limiting Maximum Visual Acuity<sup>2</sup>*

It can be seen from the data already presented that at intensities above about 1000 photons the illumination is no longer a controlling factor for visual acuity. What then does set the limit to the resolving power of the eye? Hartridge (1922) advocated intensity discrimination of the retina as the limiting factor in the resolving power of the eye. He argued that because of the chromatic aberration of the eye and of the diffraction of the light by the pupil, the image of small objects on the retina is very diffuse, and that unless the object is large enough to produce at least a 10 per cent reduction in intensity on a retinal element, it could not be resolved. This question might perhaps best be discussed separately for each of two types of test object: (1) a repetitive pattern such as the grating, and (2) a single object such as the C.

<sup>2</sup> The data of this section were presented to the April, 1939, meetings of the American Physiological Society at Toronto, Canada. *Am. J. Physiol.*, 1939, 126, 627.

1. *The Grating.*—It was previously shown (Shlaer, 1937) that the intensity discrimination of the retina in the image of a grating composed of equal opaque and clear spaces could not be the limiting factor; that the pupil diameter, if small enough, could be; and that the size of the central cones was the probable ultimate limiting factor. The grating test object diffracts the light from each point of the source into a series of spectra in the same manner that ordinary diffraction gratings do, but to a smaller extent because of its coarseness. These spectra lie in the plane of the pupil. If the pupil is not large enough to transmit the zero order spectrum and one first order spectrum up to a wavelength of about  $630\text{ m}\mu$  in white light, no resolvable image is formed on the retina. It follows that when restricted regions of the spectrum are used in making these determinations, the long wavelength regions will require larger pupils to achieve maximum visual acuities, since the red end of the spectrum lies farther from the zero order spectrum.

TABLE III

*The Effect of Color on the Resolving Power of the Eye. Grating Test Object.  
Pupil Diameter 2.35 Mm. Average of Both Observers*

Color $\lambda$ in $\text{m}\mu$	Red 670	Red 625	Orange 605	Yellow 575	White	Green 535	Blue 490	Violet 450
Visual acuity	1.80	1.91	1.99	2.08	2.10	2.14	2.11	2.07
Visual acuity	Pupil diameter 3 mm, 2.09	Pupil diameter necessary to raise the red ( $670\text{ m}\mu$ ) value to maximum is 2.8 mm. Such a pupil transmits the first order spectrum up to a $\lambda$ of $750\text{ m}\mu$ at maximum visual acuity.						

This corollary was verified in the following manner. With a fixed pupil of 2.35 mm. diameter, just large enough to achieve maximal values in white light, the maximum visual acuity for each observer was determined in light filtered through each of the seven "monochromat" filters of Eastman Kodak Company in turn.<sup>3</sup> The illumination in every case was so high that it was no longer a factor. The data are presented in Table III, each value of visual acuity being the average of all determinations of both observers. Each determination is the average of eight readings, two in each of the four different meridians used with the grating, and was made at least twice and on different days for each observer. It can be seen at a glance that the expected reduction in visual acuity at the red end of the spectrum is realized. Maximum values are not achieved in light of longer wavelength than the yellow ( $\lambda = 575\text{ m}\mu$ ).

The maximum visual acuity in the extreme red ( $\lambda = 670\text{ m}\mu$ ) is only 1.8, a loss of about 15 per cent from the average maximum of 2.1 when the pupil is not limiting. Since maximum visual acuity is directly proportional to pupil

<sup>3</sup> The authors are indebted to Dr. Joseph Mandelbaum, who assisted in recording some of these measurements.

diameter when it is limiting, it follows that an increase in the pupil of 15 per cent would eliminate it as the limiting factor. Such a pupil is 2.8 mm. in diameter. Using a pupil of 3 mm. diameter, we found the maximum visual acuity to be 2.09, which is experimentally the same as the expected 2.1.

With white light (tungsten of color temperature about 2700° K) a pupil of 2.3 mm. gave maximal visual acuities. Under these conditions the pupil transmits the first order spectrum to about 630 m $\mu$ . One may consider that when the excluded portion of the spectrum fails to interfere optically with certain transmitted radiation, an equivalent brightness is spread over the dark lines in the retinal image and thus lowers the contrast. However, the deterioration of the image on the retina by the exclusion of all wavelengths longer than 630 m $\mu$  is just not enough to lower the resolving power of the eye as set by the size of the retinal mosaic and the intensity discrimination ability of the cones. When the red filter, which absorbs all light shorter than 640 m $\mu$ , is used, a pupil of 2.35 mm. gives a maximum visual acuity of 1.8 and under these conditions the first order spectrum up to a wavelength of about 750 m $\mu$  is transmitted. The same amount of the spectrum is transmitted when the pupil is 2.8 mm. and visual acuity is maximal at 2.1. It therefore seems that for the normal eye in tungsten light filtered through the Eastman Wratten filter No. 70, the long wave limit of useful light is at 750 m $\mu$ .

2. *The C*—The fact that the relationship between visual acuity and illumination when the C is the test object is identical with the relationship between intensity discrimination and illumination is suggestive that perhaps Hartridge's notion is valid for single objects. However, the difficulty of computing the precise distribution of light in the image of the C prevents a quantitative evaluation of this idea.

This led Hecht and Mintz (1939) to investigate this relationship with a single line as a test object. They found that the intensity difference in the retinal image of their test object, as computed from the diffraction of a 3 mm. pupil, was directly proportional to the size of the line (and therefore to the visual acuity) for the entire cone range; and that the minimum difference that a single row of cones can detect was of the order of 1 per cent, which is very nearly the best intensity discrimination of which the retina is capable. They concluded that for their test object the intensity discrimination capabilities of the retina at all intensities determine the visual acuity, and suggested that perhaps the same might be true for all single objects such as the hook and broken circle.

It follows that when the visual acuity of the eye is controlled by the intensity discrimination of the retina, any increase in the intensity difference in the retinal image will result in a rise in visual acuity. Such increases in the contrast can be produced by two means: (1) homogeneous light which eliminates chromatic fringes in the image, and (2) an increase in the pupil diameter which decreases the diameter of the diffraction circle. Preliminary rough measure-

ments showed this to be true. A  $4\ \mu$  wire at 1 meter cannot be seen in white light with a 2 mm. pupil, but can be seen in monochromatic light with a 2.3 mm. pupil.

The effect of improving the retinal image of the C by the above means was then determined. The data are presented in Table IV. Each value of visual acuity is the average of four successive readings and all values in each horizontal row were taken at one continuous sitting. It is apparent that no improvement in visual acuity results from either monochromatic light or a 3 mm. pupil or both. Therefore, the intensity discrimination of the retina is probably not the limiting factor for the C at maximal intensities.

A better indication that at the higher intensities the situation with the C is not similar to that with the wire is given by the following experiment. A half-silvered mirror was mounted behind the field lens (F.L. in the apparatus

TABLE IV  
*The Effect of Pupil Diameter and Color on the Resolving  
Power of the Eye. C Test Object*

$\lambda$ (m $\mu$ )	670—Red		575—Yellow		White		490—Blue	
Pupil diameter, mm.	2	3	2	3	2	3	2	3
Visual acuity (E. L. S.)	2.56				2.54		2.56	
	2.56	2.51			2.55	2.56	2.52	2.48
Visual acuity (A. M. C.)	2.78		2.76		2.78		2.79	
	2.76	2.73			2.78	2.79		

diagram, Fig. 1, Shlaer, 1937), as viewed by the observer, and at  $45^\circ$  to the optic axis of the instrument. An auxiliary lamp, lens, and neutral wedge were then mounted at right angles to the optic axis of the instrument so that an image of the auxiliary source was formed at the pupil of the instrument by means of the auxiliary and field lenses. The illumination of the center field as viewed by the observer through the pupil consisted of two superimposed parts, one from each of the two sources. By diaphragming opposite halves of each of these two beams at the mirror it became possible to adjust the brightness of them to equality in a photometric match by means of the wedge. The brightness of the central field when these half field diaphragms were removed consisted of two equal parts, one from the regular instrument and the other from the auxiliary equipment.

When a test object is inserted in the regular manner and viewed through the pupil, it becomes equivalent to having a test object whose brightness, instead of being zero, is exactly one-half the total field brightness. In other words, it is like a test chart in which the letters are printed in light grey ink having a reflection factor of one-half that of the white card background. In the retinal

image this is equivalent to reducing the intensity discrimination fraction by somewhat more than half. Suppose that the situation on the retina when only the main instrument light is on is as follows: the intensity on a cone in the general field is 50, the intensity on a cone in the image of the circle is 40, and the intensity on a cone in the image of the break in the circle is 45. The discrimination ratio between the break and the rest of the circle is 5 parts in 40. When the auxiliary lamp is turned on, the intensity of the field becomes 100; that of the circle, 90; and that of the break, 95. The ratio now is 5 parts in 90 which is less than half the previous fraction. The smaller the discrimination fraction, the nearer the second ratio is to half the first.

If maximal visual acuity at the highest intensities is determined by intensity discrimination for the C as it is for the single line, then the maximum visual

TABLE V  
*The Effect of Diluting the Contrast of the Test Object on the  
Resolving Power of the Eye. C Test Object*

Color	Test object brightness as per cent of field	Pupil diameter	E. L. S.				A. M. C.	
			Visual acuity	Loss in visual acuity	Visual acuity	Loss in visual acuity	Visual acuity	Loss in visual acuity
		mm.		per cent		per cent		per cent
White . . . . .	0	2	2.59	0.0	2.62	0.0	2.59	0.0
White . . . . .	50	2	2.39	7.7	2.31	11.8	2.24	9.7
White.. . . .	50	3			2.31	11.8		
Blue . . . . .	50	2	2.39	7.7	2.31	11.8		
Yellow. . . . .	50	2	2.47	4.6	2.33	11.1		
Yellow . . . . .	50	3	2.46	5.0	2.46	6.1		

acuity should fall to half when this half-field-brightness test object is used. An examination of the data in the first two lines of Table V will show that such is not the case, a maximum drop of only 12 per cent having occurred. Each datum of Table V is the average of four successive determinations, and the data in each column were taken during a single continuous sitting.

When a drop in maximal visual acuity occurs as a result of the diluted test object, visual acuity must be limited by the intensity discrimination of the retina. The visual acuity should then be improved by the same two means, namely, homogeneous light and a larger pupil, that improve the resolution of the eye for a wire. The lower portion of Table V records the effect of these two variables. These measurements were made with only E.L.S. as observer on 2 different days. It is apparent that the larger pupil alone is without effect, that monochromatic blue light alone is also without effect, but that the combination of monochromatic yellow light and large pupil has a large effect, raising the visual acuity almost half way to normal. The measurements with yellow



light and the small pupil are inconsistent for the two runs, the effect being large in the first and small in the second. These measurements are not extensive enough to permit the determination of any precise relationship, but they are adequate to show that maximal visual acuity with the C test object can be improved by means of homogeneous light and a larger pupil when the intensity discrimination of the retina is the limiting factor.

It must be emphasized that the experiments in this section are concerned only with maximal visual acuity where the illumination is so high that it is no longer a factor. It is certain that under these conditions something other than the intensity discrimination of the retina is the limiting factor. This factor may very well be the diameter of the foveal cones since the geometric size of the break in the C is only a little smaller than that. What may be the controlling factor or factors at submaximal values of visual acuity is still uncertain.

## V

### SUMMARY

The relation between visual acuity and illumination was measured in red and blue light, using a broken circle or C and a grating as test objects.

The red light data fall on single continuous curves representing pure cone vision. The blue light data fall on two distinct curves with a transition at about 0.03 photons. Values below this intensity represent pure rod vision. Those immediately above represent the cooperative activity of rods and cones, and yield higher visual acuities than either. Pure cone vision in this intensity region is given by central fixation (C test object). All the rest of the values above this transition region represent pure cone vision. In blue light the rod data with the C lie about 1.5 log units lower on the intensity axis (cone scale) than they do in white light, while with the grating they lie about 1.0 log unit lower than in white light.

Both the pure rod and cone data with the C test object are precisely described by one form of the stationary state equation. With the grating test object and a non-limiting pupil, the pure rod and cone data are described by another form of the same equation in which the curve is half as steep. The introduction of a small pupil, which limits maximum visual acuity, makes the relation between visual acuity and illumination appear steeper.

Determinations of maximum visual acuities under a variety of conditions show that for the grating the pupil has to be larger, the longer the wavelength of the light, in order for the pupil not to be the limiting factor.

Similar measurements with the C show that when intensity discrimination at the retina is experimentally made the limiting factor in resolution, visual acuity is improved by conditions designed to increase image contrast. However, intensity discrimination cannot be the limiting factor for the ordinary test object resolution because the conditions designed to improve image

contrast do not improve maximum visual acuity, while those which reduce image contrast do not produce proportional reductions of visual acuity.

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# SOME PROPERTIES OF CRYSTALLINE GUINEA PIG HEMOGLOBIN

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Several lines of evidence lead to the conception of wet protein crystals as made up of protein molecules with interstices between them that are large and filled with loosely held solvent molecules. Some of the evidence leading to this conclusion may be briefly summarized: (a) the high water content of protein crystals, early noted by Sørensen (1), who estimated that over 20 per cent by weight of egg albumen crystals is water; (b) the ease of water loss under slightly diminished aqueous tension as observed by Katz (2); (c) the change in density when suspended in solutions of different osmotic pressures as studied by Adair and Adair (3); (d) the staining of protein crystals with dyes; (e) x-ray data on wet pepsin crystals, from which Bernal and Crowfoot (4) infer that the protein molecules are relatively dense globular bodies separated by relatively large spaces which contain water.

We wish to present further evidence in support of this idea, and also to describe several other properties of crystalline guinea pig hemoglobin.

## I

*Action of  $K_3Fe(CN)_6$  on Crystalline Oxyhemoglobin.*—When a suspension of crystals of guinea pig oxyhemoglobin is treated under the microscope with  $K_3Fe(CN)_6$  solution, the red crystals are observed to become yellow-brown while the crystal shape remains intact. (Other oxidizing agents of high potential such as quinone or  $KMnO_4$  also bring about this change in color.) With the Abbe microspectroscope the yellow-brown color of the crystals was identified as Mth.<sup>1</sup> This experiment suggested that ferricyanide was penetrating into the crystal to react with the iron in  $HbO_2$ .

In order to determine whether all of the  $HbO_2$  in the crystals could be converted into Mth, a quantitative study with Warburg manometers was made of the  $O_2$  released when  $HbO_2$  is acted upon by ferricyanide. Freshly prepared

<sup>1</sup> Abbreviations used.

Hb = ferrous hemoglobin

$HbO_2$  = ferrous oxyhemoglobin

Mth = ferri hemoglobin or methemoglobin

washed crystals of guinea pig  $\text{HbO}_2$  were suspended in  $\text{M}/30$  phosphate buffer pH 7.0 and carefully pipetted into Warburg vessels that had two side arms containing the ferricyanide. The ferricyanide was added in two successive portions. The first portion, 0.05 cc., was sufficient to theoretically react with only

TABLE I

*Recovery of  $\text{O}_2$  Released by Ferricyanide from Crystals of  $\text{HbO}_2$* 

Each vessel contained 2.0 cc. of  $\text{HbO}_2$  crystals\* suspended in  $\text{M}/30$  Spørensen's  $\text{PO}_4$  buffer, pH 7.0 at  $29.8^\circ\text{C}$ . (1). The dry weight of  $\text{HbO}_2$  per cc. of suspension is 0.0493 gm.  $\approx$  66 c. mm.  $\text{O}_2$  when quantitatively converted into methemoglobin with ferricyanide. If recovery of  $\text{O}_2$  is 100 per cent then 0.100 cc. of 0.05  $\text{M}$   $\text{K}_3\text{Fe}(\text{CN})_6$  should release 112 c. mm.  $\text{O}_2$  from  $\text{HbO}_2$ .

Vessel No.	0.05 $\text{M}$ $\text{K}_3\text{Fe}(\text{CN})_6$ added		$\text{O}_2$ produced		Recovery of $\text{O}_2$
	cc.		c. mm.		per cent
1	0.05	0.15	54.6	127.2	97.3
	0.10		72.6		96.3
1A	0.05	0.10	51.8	104.8	92.5
	0.05		53.0		94.0
3A	0.30			133	100
5A	0.05	0.25	54.6	137.2	97.5
	0.20		82.6		105
7	0.05	0.25	53.0	134.4	94.6
	0.20		81.4		102
8	0.05	0.25	57.4	135.4	102.5
	0.20		78.0		102
1A'	0.20	+ Veronal sodium†	135.0		102
			135.0		102

\* The uniformity of pipetting the crystal suspension into Warburg vessels gives rise to a maximum error of 2 per cent, as shown by the dry weight of three samples of  $\text{HbO}_2$  suspension pipetted under the same conditions: 0.1190 gm.; 0.1220 gm.; 0.1181 gm.

† 0.20 cc. of a saturated sodium veronal solution was added from the side arm to make the solution sufficiently alkaline to dissolve the crystals. It is seen that no additional oxygen is recovered when the hemoglobin is in solution.

42 per cent of the total oxyhemoglobin. It is seen from Table I that this reaction was quantitative and corresponded stoichiometrically to the amount of ferricyanide added. From Table II it is seen that 85 per cent of the resulting Mth was in the crystalline state at the end of the experiment. Therefore the ferricyanide molecules must have penetrated the crystals and reacted with all the prosthetic groups of oxyhemoglobin. Considering the size and shape

of our crystals, this would mean that some molecules of ferricyanide in order to get to the center of the crystals would have had to penetrate a distance equivalent to the diameter of 1,500 hemoglobin molecules. The conclusion is obvious that there must be interstices in the crystals large enough to permit the movement of ferricyanide into them.

The speed of free diffusion of ferricyanide as calculated from the Stokes-Einstein equation under the conditions of our experiment is approximately  $4 \times 10^{-8}$  cm. per second. It was calculated that if the crystal offered no appreciable resistance to diffusion of ferricyanide, the whole journey through liquid and crystal would take the maximum time of 2 to 3 seconds. The maximum time experimentally determined in the manometer for complete

TABLE II

*Solubility of the Crystals Used, at 29.8°C.*

To each of two centrifuge tubes with conical ends, add 2.5 cc.  $\text{HbO}_2$  crystal suspension in  $\text{M}/30 \text{ PO}_4$  buffer pH 7.0. To tube 1 add 0.2 cc.  $\text{H}_2\text{O}$ ; to tube 2 add 0.2 cc. of 0.05  $\text{M K}_3\text{Fe}(\text{CN})_6$ . These are kept at 29.8° for 2 hours. They are then centrifuged sharply, the supernatant liquid decanted, and adhering liquid wiped away. Tube 1 now contains crystals of  $\text{HbO}_2$  and tube 2 contains crystals of Mth. The crystals were weighed wet, and then dried at 110°C. to constant weight.

Tube No.	Dry weight of Hb in 2.5 cc.	Weight of crystals after decanting		Final amount of Hb suspension in crystal- line state
		Wet	Dry	
1	0.1227 g.	0.2874	0.1097	per cent 89
2	0.1227	0.2790	0.1053	85

release of  $\text{O}_2$  was 2 to 3 minutes. Undoubtedly a considerable portion of this time is due to the slowness of equilibration between gas and liquid phase in the manometer vessels. If the ferricyanide ions are to diffuse somewhat readily into the crystals of oxyhemoglobin the interstices must be at least 1.5 to 2.0 times the diameter of the ion, or from 6 to 10 Å.

Let us assume that the hemoglobin molecules are spheres and that they are arranged in closest hexagonal packing. It may be readily calculated that the volume of space between the spherical molecules occupies about 25 per cent of the total volume of the cube, or if filled with water this would mean that 25 per cent by volume or 20 per cent by weight of the wet crystal is water. These are, of course, minimum figures based on the assumption of closest packing and spherical molecules. The distance between the spheres would be just sufficient to permit ferricyanide movement into the spaces. It may be predicted that optically isotropic protein crystals will be found to contain at least this amount of loosely held water between the molecules.

### *Preparations Used*

The tetrahedral crystals of  $\text{HbO}_2$  were prepared from guinea pigs, bled by heart puncture into warm sodium citrate solution. The blood was washed twice with warm isotonic saline by centrifugation. To lake the cells three volumes of water were added to one of packed cells. Crystals started to form at once. The laked blood was kept at  $37^\circ\text{C}$ . for 2 hours in a 2-inch thick layer in a beaker and then centrifuged slowly for a short time. In this way only the large crystals are deposited, the supernatant solution being discarded. The crystals are suspended in water and centrifuged slowly, the process being repeated two more times. The crystals thus prepared were kept under a thin layer of water at  $1^\circ\text{C}$ . overnight. The next morning 10 cc. of the packed crystals were added to 45 cc. of Sørensen buffer ( $\text{M}/15$  in phosphate) pH 7.0 and the suspension finally made up to 90 cc. with water. These guinea pig  $\text{HbO}_2$  crystals were found to be practically free of catalase activity.

Mth was prepared from  $\text{HbO}_2$  crystals by treatment with a slight excess of ferricyanide and subsequently washing the crystals three times by means of centrifuging, and then dialyzing them against water for 24 hours. A solution of Mth was then prepared by making the cold suspension slightly acid with dilute acetate buffer and bringing the solution to neutrality with  $\text{M}/15$  disodium phosphate.

### II

*Location of the Prosthetic Groups.*—A corollary of the quantitative oxidation of the hemes by ferricyanide appears to be that the heme groups are on the surface of the globin molecules, otherwise one would have to assume penetration of ferricyanide into the molecules. The latter is made unlikely because of the closeness with which the peptide chains are packed within the molecule. Mirsky (5) has offered this juxtaposition of peptide chains as an explanation for the non-detection of  $-\text{SH}$  groups in some native proteins by means of ferricyanide. The data presented in Table I show that the hemes react quantitatively with the ferricyanide added. If some ferricyanide had oxidized  $-\text{SH}$  groups before it oxidized the heme groups then a lower  $\text{O}_2$  production per ferricyanide molecule would have been expected. For example, assuming only one free  $-\text{SH}$  group reacting per hemoglobin molecule, one would expect to recover only 75 per cent of the  $\text{O}_2$ .

In addition to being localized on the surface of the globin, the four hemes per globin must be facing the spaces between the molecules, or in other words, the hemes are not the points of closest contact between the molecules.

### III

*Further Evidence of Permeability of  $\text{HbO}_2$  Crystals.*—Hydrosulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) may also be shown to penetrate the crystal of  $\text{HbO}_2$  removing the oxygen and producing Hb. This becomes evident by diminishing the solubility of Hb with ammonium or sodium sulfate. When  $\text{HbO}_2$  crystals were suspended in 50 per cent saturated ammonium sulfate and hydrosulfite added, the crystals could be seen to turn violet under the microscope and the Hb band became

visible at the same time that the  $\text{HbO}_2$  bands vanished in the micro spectro-scope. These violet crystals go into solution within a few minutes. If the concentration of ammonium sulfate is increased to near saturation many of the purple crystals do not dissolve.

The staining of protein crystals with dyes may also represent penetration of the dye molecules into the crystal spaces. Several dyes in aqueous solution were added to  $\text{HbO}_2$  crystals and examined microscopically. Due to the intense color of the crystals it is difficult to judge their penetration. Crystal violet, however, stained the crystals intensely but not methyl green. Catalase prisms and plates were better objects because of their negligible color (6). Methylene blue and toluidine blue stained them faintly, crystal violet very strongly, and methyl green very faintly. If the dye concentration, is properly chosen the crystals will become deeply colored and the solution colorless. The staining is not merely a surface adsorption since thicker crystals take up a very much deeper stain than thinner ones.

#### IV

*The Relative Solubilities of Hb,  $\text{HbO}_2$ , and Mth.*— $\text{HbO}_2$ , as isolated from guinea pig blood is rather insoluble at room temperature. A suspension kept in  $\text{M}/30 \text{ PO}_4$  buffer, pH 7.0, for 1 day at  $1^\circ\text{C}$ . and then for 3 hours at  $30^\circ$  was 89 per cent in the crystalline state. Mth is slightly more soluble, 85 per cent remaining in the crystalline state under these conditions. Hb, however, is very soluble at this pH.

When crystals of  $\text{HbO}_2$  are watched under the microscope, as hydrosulfite diffuses to them, they are seen to melt away rapidly while still retaining their tetrahedral shape. At the same time the Hb band becomes visible. A thick suspension of  $\text{HbO}_2$  crystals is a muddy red-brown. When treated with hydrosulfite it becomes a clear deep purple; on shaking with air it gradually becomes red, then cloudy, and begins to deposit crystals all within 1 to 2 minutes. Here is an example, then, of an aggregation and dissolution brought about by the mere addition or removal of oxygen.

#### V

*The  $\text{O}_2$ - $\text{HbO}_2$  Equilibrium in Crystalline  $\text{HbO}_2$ .*—The removal of  $\text{O}_2$  from a solution of  $\text{HbO}_2$  may be readily accomplished by lowering the pressure of  $\text{O}_2$ . Under our conditions of evacuation from a Thunberg tube with an oil pump, it required less than 2 minutes to completely convert the dilute  $\text{HbO}_2$  solution to Hb. However, if a suspension of  $\text{HbO}_2$  crystals is treated in the same manner as the  $\text{HbO}_2$  solution, then no appreciable  $\text{O}_2$  can be removed even after 15 minutes of constant pumping, although the pressure was 15 mm. to 5 mm. of mercury (decreasing as the temperature of the suspension decreased due to evaporation).

The following experiments show that the oxygen of  $\text{HbO}_2$  crystals can be



removed only by considerably decreasing the oxygen tension: (a) Bubbling either  $H_2$  or  $N_2$  gases (free from  $O_2$ ) through a suspension of  $HbO_2$  crystals at  $30^\circ$  for 20 to 30 minutes completely converted them to Hb solution. (b) Removal of  $O_2$  by hydrosulfite:  $HbO_2$  crystals in  $M/15$   $PO_4$  buffer pH 7 were placed in a Thunberg tube and evacuated. A minute amount of hydrosulfite was then added from a side arm. Within 1 to 2 minutes the suspension became a solution of Hb. The pH after the experiment was 6.9. (c) Removal of  $O_2$  by activated hydrogen: A suspension of  $HbO_2$  crystals in the presence of traces of methylene blue or catalase was treated with  $H_2$  + colloidal Pd. Within 1 to 2 minutes this suspension had been completely converted to a solution of Hb. (d) Replacement of  $O_2$  by CO: A suspension of  $HbO_2$  crystals was treated with pure CO gas for 5 minutes. The CO replaced the  $O_2$  in the crystals without changing their shape, since on the addition of a small amount of hydrosulfite no Hb results and the crystals remained intact. On the addition of a large excess of hydrosulfite, the crystals began to dissolve very slowly and unevenly, this being due to a decrease in pH.

The firmer binding of  $O_2$  in  $HbO_2$  crystals, as contrasted to  $HbO_2$  in solution, may indicate that the coordination of the molecule in the crystal has diminished the competition of other groups than  $O_2$  for the iron. The  $HbO_2$  crystals can be readily dissolved, away from the isoelectric point, and in the dissolved state the  $HbO_2$  molecules readily give up their oxygen on diminishing the  $O_2$  tension. The firm binding of  $O_2$  in  $HbO_2$  crystals would obviously prevent hemoglobin in the crystalline state from functioning as a readily reversible oxygen carrier.

## VI

*Action of  $H_2O_2$  on  $HbO_2$ .*—The crystals of  $HbO_2$  of the guinea pig, crystallized once, are practically free from catalase. On addition of a few drops of 0.5 per cent  $H_2O_2$  to a suspension of  $HbO_2$  crystals, the two prominent bands at 578 and 540 become faint, the 578 band appears to broaden, spread out to 590, and is relatively stronger than the 540 band, while the original crystalline shape remains unchanged. This compound has the same absorption bands as that of  $H_2O_2$ -methemoglobin solution first described by Keilin and Hartree (7).

## VII

*Action of Activated Hydrogen on Mth.*—Methemoglobin either in solution or in crystalline suspension at pH 7.0 in  $PO_4$  buffer is very slowly reduced by activated hydrogen. Mth plus a trace of catalase and caprylic alcohol was bubbled with hydrogen for 3 minutes and then 2 drops of colloidal Pd added. The bubbling was continued for 15 minutes during which time only traces may have been reduced as observed spectroscopically in comparison with the control.

This slow interaction may be partially explained by the fact that we are dealing with two colloids. If a trace of methylene blue or rosindulin GG is

added to the Pd-H<sub>2</sub> solution containing Mth plus a trace of catalase, then reduction to ferrohemoglobin is complete in 1 minute in the case of the solution, and reduction and solution of Mth crystals is complete in 2 to 3 minutes. The methylene blue molecules being small and reversibly oxidized act as intermediary between the two colloids.

This is in agreement with the fact that also the electron exchange between hemoglobin and the bright Pt electrode is so sluggish that potentials are only poorly established.

## VIII

*The Action of Activated Hydrogen on HbO<sub>2</sub>.*—One of the intermediates produced in the reduction of O<sub>2</sub> either as such or in the form of HbO<sub>2</sub>, by activated Pd-H<sub>2</sub> is H<sub>2</sub>O<sub>2</sub>. This may be shown by the following experiment. HbO<sub>2</sub> either in solution or as crystal suspension in PO<sub>4</sub> buffer pH 7, was treated with Pd-H<sub>2</sub>. Within 45 seconds a band at 630 mμ had arisen and also the H<sub>2</sub>O<sub>2</sub>-Mth bands had appeared. The 630 band was identified as Mth by forming the Mth-NaF compound. If a trace of catalase is added to the HbO<sub>2</sub> solution, then Pd-H<sub>2</sub> added, the Hb is formed within 1 minute and in the case of the crystalline HbO<sub>2</sub>, removal of O<sub>2</sub> and solution of crystals occurred in 1 to 3 minutes. No Mth band was observed.

Instead of using catalase to destroy the H<sub>2</sub>O<sub>2</sub>, a trace of methylene blue was used with identical results. Within 3 seconds after PdH<sub>2</sub> was added, the dye was decolorized and within 20 to 40 seconds the HbO<sub>2</sub> bands had vanished. Methylene blue may act here in three ways. Its reduced form may be oxidized by H<sub>2</sub>O<sub>2</sub> thus destroying some peroxide; it may act on any Mth that might have arisen from the action of peroxide on Hb; and it may react with the minute amount of O<sub>2</sub> in solution.

## SUMMARY

1. Guinea pig hemoglobin crystals are shown to be readily permeable to ferricyanide and hydrosulfite, indicating the presence of interstices between the protein molecules of the crystal.

2. The assumptions of closest hexagonal packing and of spherical molecules of HbO<sub>2</sub> lead to a crystal lattice having interstices between the molecules which represent 25 per cent by volume of the crystal. These spaces would be just large enough at their narrowest junctures to permit ferricyanide ions to diffuse through them. If these spaces were filled with water, then 20 per cent by weight of the crystals would be water.

3. The hemes are on the surface of the globin and are arranged facing the interstices between the molecules of the lattice.

4. The binding of O<sub>2</sub> in HbO<sub>2</sub> is stronger in the crystal lattice than in solution.

5. Hydrogen, activated with colloidal palladium, will not reduce ferrihemoglobin except in the presence of a redox dye.

6. In the reduction of  $O_2$  by activated hydrogen,  $H_2O_2$  can be demonstrated by the formation of the  $H_2O_2$ -ferrihemoglobin spectrum.

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# THE PHOTOSYNTHETIC EFFICIENCY OF PHYCOCYANIN IN CHROOCOCCUS, AND THE PROBLEM OF CAROTENOID PARTICIPATION IN PHOTOSYNTHESIS

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## I

Chlorophyll in plant cells is regularly accompanied by other pigments. For many years there has been speculation as to whether the light absorbed by these so called accessory pigments is utilized for photosynthesis. Engelmann's skillful experiments (1883) on algae of various colors, with motile bacteria as an indicator of photosynthetic activity, have been the basis of a general belief that the light absorbed by certain accessory pigments (particularly fucoxanthin, phycoerythrin, and phycocyanin) is available for photosynthesis. This belief, although criticized by some who obtained conflicting evidence, has been strengthened by photosynthesis measurements with technique allegedly superior to that of Engelmann (*cf.* Harder, 1923). But a critical examination of both early and later work on this subject shows that the evidence is inconclusive, chiefly because the relative amounts of light absorbed in different wave lengths were not established. Comparison of the photosynthetic activity in different wave lengths can be decisive in establishing the part played by accessory pigments only if the absorbed energy in each wave length is known. Recent attention to the problem of the quantum yield of photosynthesis has resulted in development of techniques well suited to investigation of the activity of accessory pigments. Dutton and Manning (1941) have compared light absorption and photosynthetic activity in the diatom *Nitzschia closterium* in different parts of the spectrum, and found good evidence that the energy absorbed by the carotenoids, especially fucoxanthin, is used in photosynthesis. Recent measurements of the quantum yield of photosynthesis for various species of algae (Emerson and Lewis, 1941, p. 803) gave strong indication that the phycocyanin of the blue-green alga *Chroococcus* is active in photosynthesis. These measurements were all made in sodium light,  $\lambda = 589 \text{ m}\mu$ . In the green algae, practically all the absorbed energy of this wave length is absorbed by chlorophyll, but in the case of *Chroococcus* the phycocyanin is responsible for a large part of the total energy absorbed. Since the quantum yield for *Chroococcus* photosynthesis was in good agreement with the yields for green algae

it seemed probable that the energy absorbed by phycocyanin must be available for photosynthesis. In the present communication we report more conclusive evidence that this is the case. The energy absorbed by phycocyanin is used for photosynthesis with an efficiency closely approximating that of chlorophyll. Our results give slight indications of photochemical activity on the part of the carotenoids as well, but it appears that any photosynthetic yield from light absorbed by these pigments must be relatively low.

## II

### *Material and Experimental Methods*

The alga, a small species of *Chroococcus*, was obtained in pure culture from C. B. van Niel of Stanford University. The cells are about 2.5 to 3  $\mu$  in diameter (some-what smaller than *Chlorella*). They are encased in a gelatinous sheath about half as thick as their own diameter. In spite of the sheath, cultures grow without serious clumping, and an inoculum of 5 c.mm. cells in 200 ml. of culture medium produces a growth of about 150 c.mm. cells in from 5 to 7 days, at a temperature of 30°C. The cultures are grown in continuous light from four closely spaced 60 watt incandescent lamps at a distance of 20 cm. from the bottoms of the culture flasks.

The composition of the culture medium is as follows:

per liter glass-distilled water,

MgSO <sub>4</sub> 7 H <sub>2</sub> O.....	0.25 gm.
KH <sub>2</sub> PO <sub>4</sub> .....	1.00 "
KNO <sub>3</sub> .....	1.00 "
Na <sub>2</sub> CO <sub>3</sub> (anhydrous).....	1.50 "
Ca(NO <sub>3</sub> ) <sub>2</sub> .....	0.025 "
NH <sub>4</sub> Cl.....	0.050 "
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> .....	0.004 "

Manganese, boron, zinc, copper, and molybdenum were added from a solution of their salts, to give concentrations of 2, 1, 0.05, 0.01, and 0.01 parts per million respectively.

The cultures fail to grow if sodium or calcium is omitted from the medium. The addition of large amounts of potassium seems to be unnecessary. Successive cultures have been grown in the above medium with sodium salts substituted for both the potassium salts, and with amounts of potassium running from 1 to 10 parts per million. Growth is poor if potassium is omitted altogether, but as little as 1 p.p.m. is sufficient to support culture growth as well as does the control medium prepared with potassium salts.

The dry weight of 100 c.mm. of *Chroococcus* cells is about 10.5 mg., as compared to a corresponding value of about 20 for *Chlorella pyrenoidosa*. The lower dry weight of *Chroococcus* is probably due to the gelatinous sheath surrounding the cells, which prevents them from packing as closely as *Chlorella* cells, so that a cubic millimeter of cells represents a smaller amount of cell material in the case of *Chroococcus*. Cell counts showed about  $4 \times 10^7$  *Chroococcus* cells per c.mm. of centrifuged volume, as compared to about  $2.3 \times 10^7$  for *Chlorella*.

For measurements of photosynthesis, the cells were centrifuged out of their culture

medium and resuspended in freshly mixed carbonate buffer (85 parts  $m/10$  bicarbonate to 15 parts  $m/10$  carbonate). In preparing this mixture for experiments with *Chroococcus*, it was necessary to use sodium, or part sodium and part potassium salts. If the mixture was made from potassium carbonate and bicarbonate, as is the usual practice for *Chlorella*, the *Chroococcus* showed a rapid decline in both photosynthesis and respiration. We made a practice of mixing sodium bicarbonate and potassium carbonate for the measurements with *Chroococcus*. In this mixture the cells maintained fairly steady rates of respiration and photosynthesis for 2 to 3 hours. The proportions of the two constituents are less critical than for *Chlorella*. *Chroococcus* photosynthesis at light saturation seems to be relatively independent of carbon dioxide concentration, even down to quite alkaline carbonate mixtures. Efforts to measure photosynthesis in acid phosphate media were unsuccessful, apparently because the photosynthesis of *Chroococcus* was inhibited by the low pH. The inhibition was reversible on returning the cells to carbonate mixture.

Rates of photosynthesis were based on manometric measurements of rate of pressure change during the second 5 minutes of alternate 10 minute periods of light and darkness. The buffering capacity of the carbonate mixture maintains a constant partial pressure of carbon dioxide, so the rates represent oxygen exchange alone. Most of the measurements were made at 20°C. The manometer and the general technique of making the measurements have already been described (Emerson and Lewis, 1939). Measurements were made with both thick and thin cell suspensions. The thick suspensions were dense enough to absorb all the incident light, so that a measurement of the incident intensity alone sufficed to establish the amount of light absorbed. The thin suspensions absorbed only about half of the incident light. The amount absorbed was determined immediately after the photosynthesis measurements, using the same cell suspension, light beam, and monochromator settings. For the measurement of light absorption, a sample of the suspension was transferred to an absorption cell of the same thickness (1.4 cm.) as the depth of the layer of cell suspension used in the manometer vessel for the photosynthesis measurements. (The effect of local irregularities in the depth of the cell suspension due to the rotary shaking of the manometer vessel was neglected.) The side walls of the absorption cell were of brass, nickel-plated on the inside to reflect scattered light. The intensity transmitted was measured by a photronic cell the active surface of which was larger than the back window of the absorption cell. The distance between active surface and absorption cell window was less than a millimeter. Thus even light which had been scattered through a considerable angle was registered by the photronic cell at nearly its full value. The incident intensity was measured, as usual, as that transmitted by a similar absorption cell filled with water instead of cell suspension.

Measurements of photosynthesis and light absorption were made in various wave length bands isolated from the light of a 1000 watt tungsten filament lamp by means of a large-aperture grating monochromator.<sup>1</sup> For measurements of photosynthesis in the red region of the spectrum, bands were used whose half-width, in-

<sup>1</sup> The monochromator was constructed with the aid of a grant from the National Research Council, and with valuable help and advice from members of the staff of the Mount Wilson Observatory. Details concerning the construction and light output of the monochromator are being prepared for publication elsewhere.

cluding nearly 75 per cent of the total energy, was from 6 to 10  $\mu$ . In the blue, because of the lower output of the lamp, wider slits were used, giving band half-widths of 15 to 20  $\mu$ . Spectral impurity due to scattered light from the various optical surfaces, principally that of the grating, was reduced by means of glass color filters which transmitted only the region of the spectrum close to the line for which the monochromator was set. Scattered light outside the band transmitted by the monochromator probably never exceeded 5 per cent of the total energy of the beam. For the quantum yield measurements, the entire light beam emitted by the monochromator was allowed to fall on the assimilating cells, or was concentrated on the sensitive surface of the bolometer for measurement of the energy received by the cell suspension. This was a modification of our former technique (Emerson and Lewis, 1939), in which only a small sample of the light beam was used for the energy measurements.

We gratefully acknowledge the cooperation of H. H. Strain in the extraction and separation of the various pigments, and in measuring the absorption of the extracts. The chlorophyll and carotenoids were extracted from the fresh, unground cells with methanol, transferred to ether, and the ether solution diluted with ethanol. The separation of pigment components and the determination of absorption spectra of the total extract in ethanol and of the chlorophyll and carotenoid fractions were carried out according to technique previously described by Strain (1938, pp. 125-132). The time allowed for saponification of the chlorophyll was 20-30 minutes. The phycocyanin was obtained in water solution by grinding the cells with silicon carbide ("crystolon") in a glass mortar at 0°C., precipitating twice with saturated ammonium sulfate, and redissolving the blue pigment by washing through a filter with distilled water.

When the carotenoids of *Chroococcus* were adsorbed on a sucrose column, the xanthophylls appeared to differ markedly from those commonly found in extracts of higher plants, and also in extracts of *Chlorella*. The carotenes, on the other hand, were apparently similar to those ordinarily encountered in leaf extracts. The unusual character of the xanthophyll-like pigments of the Cyanophyceae has been noted by several investigators (for example Tischer, 1938, and others cited by him). Unfortunately, in our work on *Chroococcus* not enough attention was devoted to the carotenoids to enable us to compare them with pigments isolated from related species by other investigators.

### III

#### EXPERIMENTAL

The peculiar usefulness of the quantum yield as evidence concerning the part played by accessory pigments is that measurements of both photosynthesis and light absorption are involved. In different regions of the spectrum, the various plant pigments absorb different proportions of the total absorbed radiation. If some components are photochemically inactive in photosynthesis, then the observed quantum yield for the total radiation absorbed by all the components will vary with the wave length. A comparison of the wave

length dependence of the observed quantum yield with the fractions of total absorbed light which are absorbed by the different components may be expected to show which pigments are photochemically active in photosynthesis, and what are their relative activities. This reasoning implies the assumption that the yield for the fraction absorbed by any one pigment is independent of wave length over the range under consideration. This assumption will be discussed presently.

### *Absorption Measurements*

Direct measurement of the fraction of light absorbed by the various pigment components in the intact cell is not possible, but we have evidence that a fairly close estimate may be made from the absorption curves of the extracted pigments. Figs. 1 and 2 show the extent of the discrepancies between the absorption characteristics of the extracted pigments and of the intact cells.

Fig. 1 shows the absorption spectra (plotted as  $\log \left( \frac{I_0}{I} \right)$ ) of the three pigment components of *Chroococcus*—chlorophyll, carotenoids, and phycocyanin. The solid curve represents the absorption of the total alcohol-soluble pigments, consisting of chlorophyll and carotenoids without phycocyanin. After saponification and removal of the chlorophyll fraction, the absorption of the carotenoids was measured. This is plotted as a broken line. The dotted line, calculated from the difference between the absorption of the carotenoids and that of the total extract, represents the absorption due to chlorophyll. From 570 to 700  $m\mu$ , where there is no absorption by carotenoids, the curve for chlorophyll is identical with the solid line for the total alcohol-soluble pigments. The absence of any maximum in the chlorophyll curve at 458  $m\mu$ , and the lack of apparent asymmetry in the red absorption band show that no appreciable amount of chlorophyll *b* is present. The absorption curve for phycocyanin dissolved in distilled water is shown by the curve drawn with alternating dots and dashes. The triangles are the observed points for our pigment, and the crosses are taken from the curve of Svedberg and Katsurai (1929), adjusted to give agreement with our curve at the maximum. The nearness of these points to our curve is evidence of the identity of the phycocyanin from *Chroococcus* and that which Svedberg and Katsurai isolated and crystallized from *Aphanizomenon flos aquae* (molecular weight about 200,000). The somewhat higher absorption of our curve in the blue may be due to the presence of a small amount of colloidal matter from the ground cells, which filtration failed to remove from the solution. In the photoelectric spectrophotometer used for all the curves in Fig. 1, even very slight light scattering would appear as absorption. For purposes of the subsequent discussion we have assumed that the absorptions measured in this way are correct. If the true values are lower



in the blue, this only adds emphasis to the discrepancy between light absorption and photosynthetic efficiency to be discussed below.

In order to compare the absorption by the pigments in the intact cells with the combined absorption of the extracted pigments, we have plotted them to-

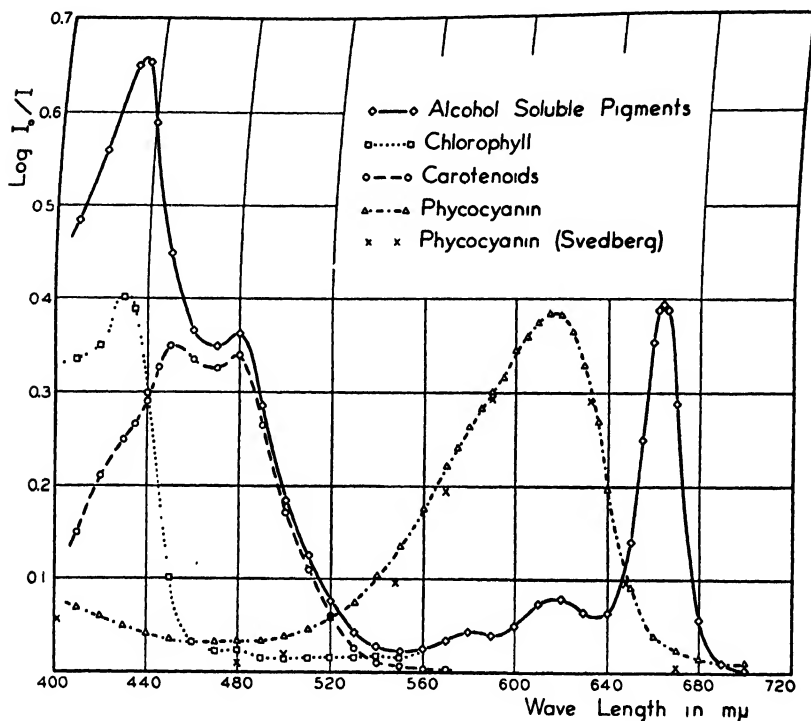


FIG. 1. The absorption spectra of the extracted pigments of *Chroococcus*. The solid line is for the total alcohol-soluble pigments, carotenoids, and chlorophyll. The broken line is for carotenoids alone, after saponification and removal of the chlorophyll. The dotted curve for chlorophyll represents the difference between the curve for the total alcohol extract and the one for carotenoids alone. In the red region, where there is no carotenoid absorption, the curve for chlorophyll is identical with the solid line for the total extract. The curve drawn with alternate dots and dashes is for an aqueous solution of phycocyanin.

gether in Fig. 2 (the two lower curves). The solid curve shows the absorption by a suspension of live cells, measured with a photronic cell by the method described in Section II. The broken curve shows the calculated combined absorption of the extracted pigments. In making this computation, certain adjustments have been made which must be kept in mind in order to make a fair comparison between the two curves. Each measured absorption curve

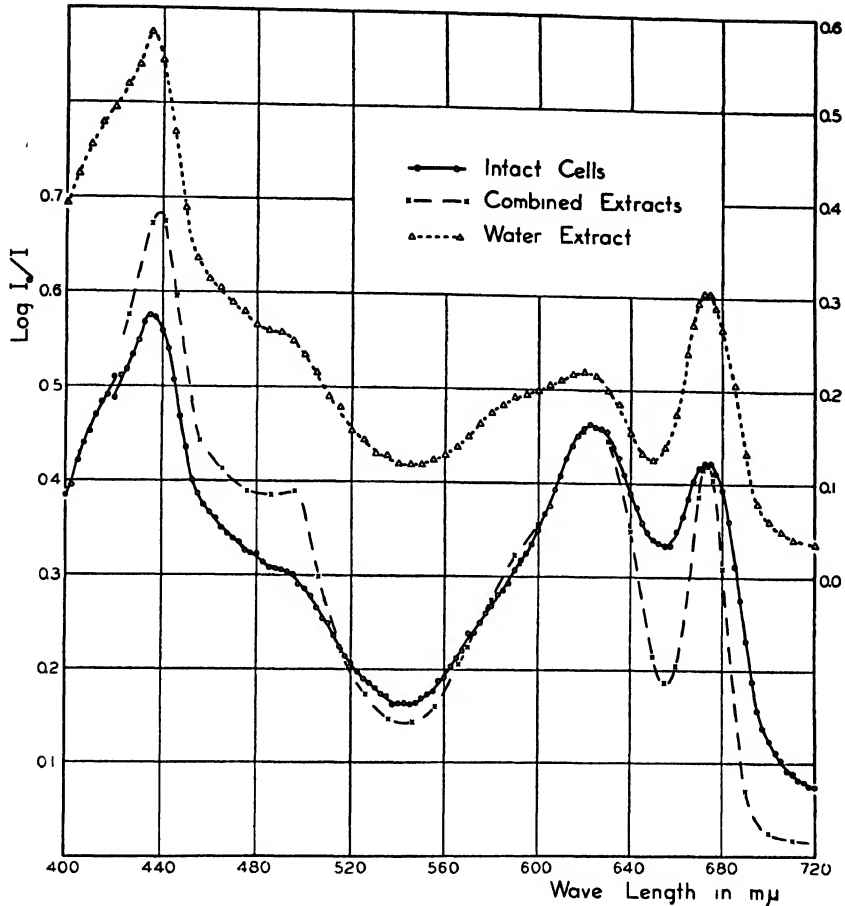


FIG. 2. Comparison of the sum of the absorption spectra of the extracted pigments, with the absorption spectrum for intact *Chroococcus* cells. The curves are based on a cell concentration of 1.26 c.mm. cells per ml. of suspension, in a layer 1.4 cm. thick. The solid curve is for the intact cells. The broken curve is for the total extracted pigments, obtained by addition of the curves for individual components shown in Fig. 1, after making adjustments in the positions of the maxima as explained in the text. The dotted curve, displaced upward on the ordinate axis to avoid confusion of the other two curves, is for an aqueous extract of ground cells. The scale for the dotted curve is on the right.

shown in Fig. 1 was shifted as a whole toward longer wave lengths an amount which appeared to make the individual maxima correspond best with the respective maxima for the live cells. In the case of chlorophyll, this shift was different in the red and blue regions, being 10 mμ in the red, and 6 in the blue

(the change being made arbitrarily at  $580\text{ m}\mu$ ). The carotenoid curve was shifted  $14\text{ m}\mu$ , and that for phycocyanin  $6\text{ m}\mu$ . The chlorophyll and carotenoid curves in Fig. 1 are directly comparable with the curve for the live cells in Fig. 2, because aliquot portions of the same culture were used, and extraction and separation were carried out quantitatively. On the assumption that Beer's law applies to our conditions, the measurements made in solution were so corrected that they apply to the same light path and concentration of pigment per unit volume as prevailed in the cell suspension. (However, the exact agreement of the curves in Fig. 2 at the red chlorophyll maximum must be considered accidental.) The extraction and separation of phycocyanin, on the other hand, were not made quantitatively. The curve in Fig. 1 represents an arbitrary concentration, chosen to give agreement between the two curves in Fig. 2 at the phycocyanin absorption maximum. This is the only adjustment involved in the calculated curve aside from the wave length shifts mentioned above.

A comparison of the curves in Fig. 2 for the observed absorption of the intact cells, and for the absorption calculated from the curves for the combined extracts (the solid and broken curves respectively), shows that in the region of carotenoid absorption the calculated curve is too high. This is perhaps an indication that these pigments absorb somewhat more in solution than in the live cells. The chlorophyll absorption band in the red is obviously much broader in the intact cells than in solution, so that for wave lengths near this band the absorption measured in solution is significantly lower than for live cells. It is not clear to what extent this applies also to the blue band of chlorophyll, and to the carotenoid band.

It is noteworthy that the absorption curve measured for a scattering suspension can give such good agreement with that of the extracted pigments in homogeneous solution. This favorable result may be due in part to the absence of plastids in the *Chroococcus* cells, and consequent simpler arrangement of pigments than prevails in the higher algae. In *Chroococcus* the pigments appear to be uniformly distributed throughout the protoplast. In the case of *Chlorella*, where the pigments are concentrated in plastids, comparison of the absorption spectrum of the live cells with that of the extracted pigments gives far less satisfactory agreement. Further evidence of the relative simplicity of pigment arrangement in *Chroococcus* is obtainable from an examination of water extracts. It is well known that water extracts from ground cells of higher green plants are difficult to analyze spectroscopically, although Smith (1941) has shown that satisfactory absorption curves can be made with extracts in aqueous digitonin or other detergents. But if *Chroococcus* cells are ground in a glass mortar with an equal volume of crystolon and then diluted with distilled water and centrifuged, a fairly clear extract of the combined pigments is obtained without resort to detergents. The absorption curve of such an extract is included in Fig. 2 (dotted curve). The sharpness of the absorption bands is comparable with that of Smith's digitonin extract. The aqueous extract was not perfectly clear,

so the absorption spectrum was measured with the photronic cell. The plotted values, displaced upward on the ordinate scale to avoid confusion of the other two curves (scale on the right), refer to a pigment concentration arbitrarily chosen to give agreement with the blue chlorophyll maximum of the live cells. The extract was prepared from a sample of the same culture used for the other two curves in Fig. 2. In the aqueous extract of pigments the positions of the maxima of the alcohol-soluble pigments remain unaltered, as compared to the corresponding maxima for the

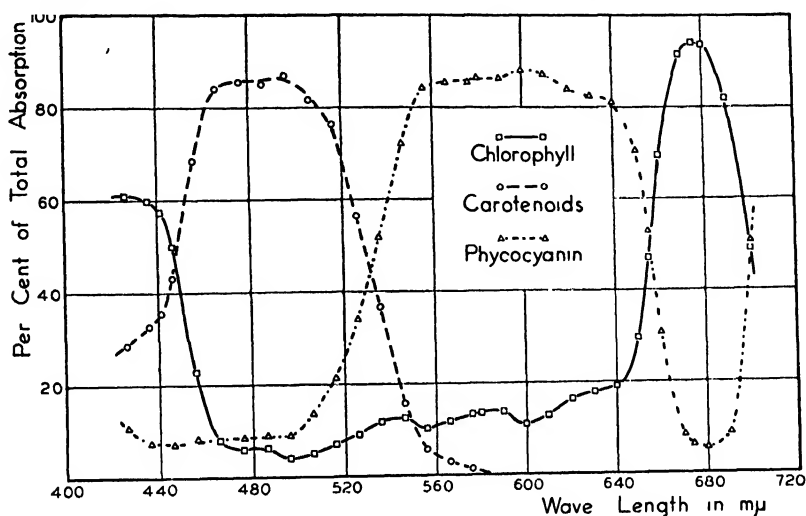


FIG. 3. These curves show what percentage of the total absorbed light is absorbed by each of the three pigment components, and how the percentage absorbed by each component varies with wave length. The solid curve is for chlorophyll, the broken curve is for the carotenoids, and the curve drawn with alternate dots and dashes is for phycocyanin. These curves are based on the measurements with extracted pigments. The values for total light absorbed were taken from the points of the broken curve in Fig. 2. To find the per cent absorbed by each component, the values plotted in Fig. 1 were used, after making the same adjustments in positions of the maxima as were used in deriving the broken curve in Fig. 2.

intact cells. The absorption band of phycocyanin, however, is shifted, opening up a deep minimum at  $650\text{ m}\mu$ , which is particularly striking when the absorption is observed visually. The secondary maximum at  $590\text{ m}\mu$ , barely visible in the isolated pigment, is quite strongly developed. This may be related to the reduced level of phycocyanin relative to the other pigments present in the aqueous extract.

The good general agreement between the two lower curves of Fig. 2 indicates that, at least in *Chroococcus*, extraction of the pigments results in no fundamental change in their absorption spectra. The data of Fig. 1 have therefore been used to calculate the per cent of the total absorbed light which is absorbed

by each of the three pigment components, using the wave length shifts mentioned above. Fig. 3 shows how the percentage of light absorbed by each component varies with wave length. The values plotted in this figure are compared in the next section with the dependence of the quantum yield on wave length, in order to show how much of the photosynthetic activity is due to light absorption by the accessory pigments.

Fig. 3 is clearly an inexact picture of the percentage of light absorbed by the pigment components in the intact cell, since the curves in Fig. 2 show certain discrepancies between intact cells and extracted pigments. Even where the combined absorption of the pigment extracts agrees well with the absorption of the cells, there is no certainty that relative absorption of the components can be calculated correctly from the measured absorption of the extracts. It is always possible that the process of extraction increases the absorption of one pigment and decreases that of another, in such a way as to produce no net change in the total absorption. While such uncertainties are a distinct limitation of the method, at present there appears to be no simple way of avoiding them.

### *Quantum Yield*

The quantum yield for *Chroococcus* photosynthesis is plotted against wave length in Fig. 4 (solid line). Eight separate runs were made, in general on different days and with cells from different cultures. The points of each set are identified by distinguishing characters on the graph. Although careful control of culture conditions made it possible to obtain cells with very nearly the same characteristics from different cultures, the reproducibility was not perfect. It was assumed that a slightly lower activity of a particular culture would lower the quantum yield at different wave lengths by the same factor, so that a uniform correction could properly be applied. One set of values in Fig. 4 has been multiplied by 1.05, and another by 1.13, to give a better fit with the other points. One culture gave yields about 20 per cent below the others, and has been omitted. The other five sets are presented as they were obtained. In some runs the series of wave lengths was gone over twice, in other cases only once. A small correction was sometimes made for the observed decline in activity of the cells during the 2 or 3 hours of a run. Of the 36 points, three, belonging to different sets, appear to be definitely out of line with the others, and have been arbitrarily omitted from consideration in drawing the curve.

Considering first the red end of the spectrum, Fig. 4 shows that the quantum yield is essentially constant from 690  $m\mu$  to about 570  $m\mu$ , aside from the small maximum at 680. It is uncertain whether this maximum is real, or is the result of a higher activity of the particular culture used for this region. The difference in photosynthetic efficiency over the entire range does not in any

case appear to be more than about 10 per cent. Fig. 3 shows that at 676  $m\mu$  phycocyanin absorbs 6 per cent of the total light absorbed, chlorophyll 94 per cent; while throughout the region from 640 to 560 the relationship is nearly reversed, with phycocyanin absorbing over 80 per cent, and chlorophyll less than 20 per cent. Even allowing wide margins for the uncertainty in estimating absorption in the live cell from the measurements on extracted pigments, it is clear that the light absorbed by phycocyanin must be available for photo-

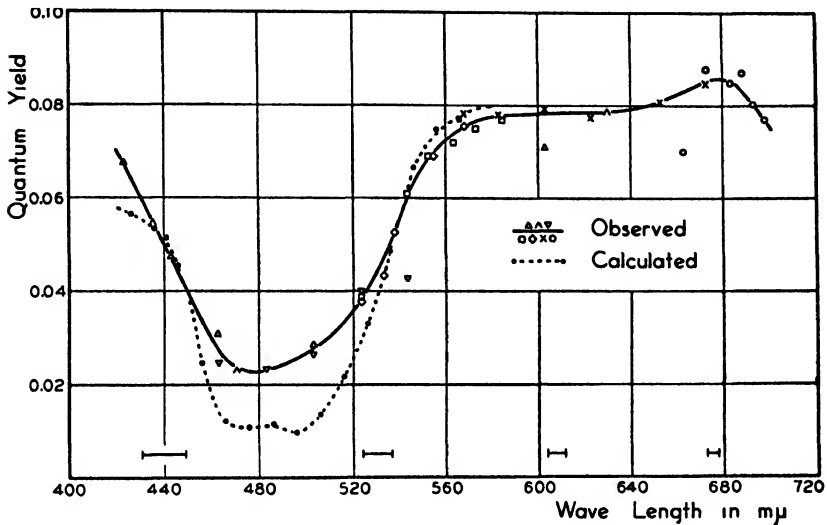


FIG. 4. The quantum yield of *Chroococcus* photosynthesis. The solid line is drawn through the experimental points, the values obtained in different runs being distinguished by different characters. The dotted curve shows the expected dependence of the quantum yield on wave length, on the assumption that the yield for light absorbed by chlorophyll and phycocyanin is 0.08 at all wave lengths, and that the light absorbed by the carotenoids is not available for photosynthesis.

synthesis with an efficiency of the same order of magnitude as that absorbed by chlorophyll.

Proceeding to the short wave length half of Fig. 4, the most obvious explanation for the sharp decline in yield beyond 570  $m\mu$  would be that the carotenoids are here absorbing light but not contributing the energy to photosynthesis. To test this hypothesis, we have calculated the expected photosynthetic yield for the total energy absorbed by the live cells, using the figures for relative absorption of the three pigment components, as plotted in Fig. 3 from the curves for the extracted pigments. The quantum yield for light absorbed by chlorophyll or by phycocyanin is assumed to be 0.08, and the yield for light absorbed by carotenoids zero. The resulting curve for yield as a function of wave

length is shown by the dotted line in Fig. 4. Qualitatively this is in fair agreement with the solid line drawn through the experimental points, a clear indication that the light absorbed by the yellow pigments is relatively ineffective in photosynthesis. But quantitatively the calculated yield is too low by a factor of two in the region of lowest efficiency. This is definitely outside the experimental error of the quantum yield measurements, and appears to be an unreasonable discrepancy to attribute to the assumptions involved in calculating the relative amounts of light absorbed by the pigment components.

On the other hand, if the above calculation is modified by assuming that the yield for light absorbed by the carotenoids is one-fifth that for chlorophyll and phycocyanin, instead of zero, then each point on the calculated curve is raised one-fifth of the distance between its present location on the dotted curve and the value 0.08. Agreement with the experimental curve is then entirely satisfactory, the minor discrepancies being less than one would expect in view of the imperfect agreement between calculated and measured absorption for the live cells shown in Fig. 2. The uncertainties involved indicate that no great significance should be attached to the precise value assumed in this case for the quantum yield for light absorbed by the carotenoids. But the good general agreement obtained suggests that a small part of the energy absorbed by the carotenoids may be available for photosynthesis.

#### *Measurements with Thin Suspensions*

As a check of the results with thick suspensions and in order to get additional information, a few runs were made with suspensions absorbing roughly half of the incident light. The photosynthesis and incident energy were measured in the same way as before, and the fraction of the incident light absorbed by the suspension was determined as a function of wave length directly after each run, in the apparatus described in Section II. The resulting values for the quantum yield were in general agreement with those obtained with totally absorbing suspensions, tending to confirm both the latter and the technique of absorption measurements. Though the absorption in different regions varied by a factor of more than two, and the quantum yield by a factor of more than three, the calculated quantum yield showed, within 10 per cent, the same dependence on wave length as the solid curve in Fig. 4.

These measurements with thin cell suspensions may also be presented in another way, to distinguish between that portion of the absorbed light which is absorbed by pigments photochemically active in photosynthesis, and that which is absorbed by inactive pigments. The usefulness of a distinction between "active" and "inactive" pigments depends in part on two simplifying assumptions. These are that the quantum yield for light absorbed by any one pigment is independent of wave length, and that the yields for the various accessory pigments are either zero or equal to the yield for light absorbed by

chlorophyll. (The possibility of generalizing the discussion to include intermediate and even varying efficiencies is obvious.)

The measurements of photosynthesis and light intensity with the thin suspensions show the yield per quantum of incident energy. We call this  $\phi'$  to distinguish it from  $\phi$ , the conventional symbol for the yield per quantum of absorbed energy. The value of  $\phi'$  varies with wave length, and depends not only on the fraction of the incident light which is absorbed by the cell suspension, but also on the fraction of the absorbed energy which is absorbed by pigments active in photosynthesis. This latter fraction is not measurable directly, but can be estimated as follows. We will use  $\phi_o$  to denote the yield for energy absorbed by chlorophyll, a pigment which we are sure is photochemically active in photosynthesis. The value of  $\phi_o$  may be assumed, or it may be measured directly at some wave length not appreciably absorbed by the other pigments present. According to the assumptions mentioned above, the value of  $\phi_o$  is independent of wave length, and applies also to the energy absorbed by any other pigments besides chlorophyll which may be active in photosynthesis. Therefore at any wave length where  $\phi'$  has been measured, we can use the value of  $\phi_o$  to find the fraction of the incident energy which is absorbed by photosynthetically active pigments. This fraction is  $\frac{\phi'}{\phi_o}$ , the "active absorption" for the particular suspension of cells on which  $\phi'$  was measured. It is to be compared with the total absorption, measured directly on the same cell suspension with the photonic cell. To express total absorption in the same units as active absorption, we will call  $I_o$  the incident energy, and  $I'$  the absorbed energy (the difference between the incident and transmitted energy). Then  $\frac{I'}{I_o}$  is the total absorption, that fraction of the incident energy which is absorbed by all the pigments present in the suspension, both active and inactive. Comparison of  $\frac{I'}{I_o}$  with  $\frac{\phi'}{\phi_o}$  should show what fraction of the absorption is attributable to active pigments and what to inactive pigments. Reference to the absorption spectra of the extracted pigments may then show which are the active and which the inactive pigments.

Fig. 5 shows a plot of measurements of both  $\frac{I'}{I_o} \times 100$  (solid curve) and  $\frac{\phi'}{\phi_o} \times 100$  (broken curve), against wave length. Three experiments are represented, each covering a different portion of the spectrum. For the run between 600 and 700 m $\mu$ , the figure of 0.075 was used for  $\phi_o$ , the yield for light absorbed by chlorophyll. This value was measured with a thick suspension of cells from the same culture as the one used for the thin-suspension measurements. For the other two runs, no direct determinations of  $\phi_o$  were made, and



a value of 0.070 was assumed, in order to bring the curves for active and total absorption roughly together at 583  $m\mu$ .

In the long wave length section of Fig. 5, the curve for total absorption shows a maximum near 670  $m\mu$  due to chlorophyll, and another one near 620  $m\mu$

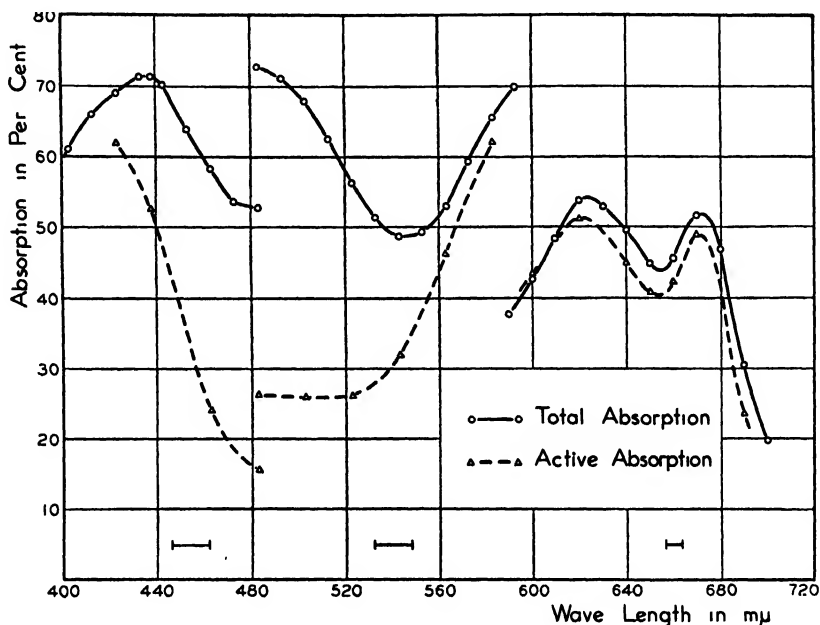


FIG. 5. Comparison of absorption spectrum and "action spectrum." The three parts of the figure represent three different runs, each covering a portion of the spectrum. For the blue region a concentration of 1.02 c.mm. cells per ml. of suspension was used, for the green region 1.67 c.mm., and for the red region 0.75 c.mm. The short horizontal line in each section shows the half-width of the band used for the run represented. The solid line shows the per cent of incident light absorbed by a suspension of live *Chroococcus* cells. The broken curve shows the per cent of the incident light which is absorbed by photosynthetically active pigments, on the assumption that all the light used for photosynthesis gives a quantum yield equal to the observed yield for the light absorbed by chlorophyll.

due to phycocyanin. The curve for active absorption shows two maxima which correspond closely to the ones for total absorption. The active absorption is nearly equal to the total absorption, showing that the same fraction of the incident energy which is absorbed is also used for photosynthesis. This is clear evidence that radiation absorbed by phycocyanin, as well as that absorbed by chlorophyll, is used for photosynthesis. There is no indication that the quantum yield for phycocyanin is any lower than for chlorophyll.

Our interpretation of this evidence, taken together with that in thick suspensions, is that the two yields are the same within 10 per cent, and probably within 5 per cent.

In the region of carotenoid absorption the curves behave quite differently. Below 560  $m\mu$ , as the total absorption turns sharply upward, the active absorption continues to decline. After going through a broad minimum from 520 to 480  $m\mu$ , it finally rises gradually as the blue absorption band of chlorophyll is approached, and at 423  $m\mu$  nearly rejoins the curve of total absorption. The area between the two curves, which from the point of view outlined above represents absorption by inactive pigments, may evidently be associated with carotenoid absorption. However, the active absorption in Fig. 5 never reaches a lower fraction of the total absorption than 29 per cent. Calculation based on absorption of the extracted pigments indicates that throughout the region from 466 to 500  $m\mu$  chlorophyll and phycocyanin together account for less than 15 per cent of the total absorption. The discrepancy, amounting to a factor of two, is as before not easily attributable to errors in the measurement of photosynthesis or radiant energy. It is possible that throughout the region of low efficiency the process of extraction alters the absorption by the pigments much more than was assumed. The discrepancy would be eliminated if we should assume that in the plant chlorophyll absorbs about three times as much, or that the carotenoids absorb only half as much, as in solution. The curves in Fig. 2 make either of these hypotheses seem unlikely, but a more moderate effect of extraction on both pigments is certainly compatible with the absorption measurements. Due to this possibility no more definite conclusions can be drawn than from the experiments with thick suspensions. Both groups of experiments suggest either that certain of the carotenoid pigments of *Chroococcus* are photochemically active in photosynthesis, or that all are active with an efficiency much lower than that of chlorophyll and phycocyanin. Any attempt to distinguish between these two possibilities by the present method would be difficult because of the similarity between the absorption spectra of the various carotenoid pigments.

In presenting the experiments with both thick and thin suspensions, we have made use of the assumption that the quantum yield for light absorbed by any one pigment is independent of wave length. It is appropriate to mention some experimental evidence in support of this assumption. In the case of the green alga *Chlorella pyrenoidosa*, which contains no phycocyanin, practically the entire absorption of light at wave lengths longer than about 570  $m\mu$  can be attributed to chlorophyll. Measurements of the quantum yield of *Chlorella* photosynthesis at different wave lengths (the results of which are being prepared for publication) have shown that from about 570 to 680  $m\mu$ , the yield is essentially constant. Franck and Herzfeld (1937, p. 251) state that the yield of chlorophyll fluorescence is constant from 400 to 650  $m\mu$ . If their

interpretation of the significance of fluorescence is correct, then constancy of the yield of fluorescence favors the conclusion that the photosynthetic yield for light absorbed by chlorophyll must be constant, not only in the region where chlorophyll alone absorbs light, where the experiments with *Chlorella* indicate that it is constant, but also at wave lengths where other pigments compete with chlorophyll for the absorption of light and make direct measurements impossible. But the extension of the assumption of constant photosynthetic yields for each pigment to organisms other than *Chlorella*, and pigments other than chlorophyll, must be regarded as only a working hypothesis.

It must be emphasized that these reservations concern only the conclusions drawn in regard to the carotenoid pigments. The experiments reported here establish beyond reasonable doubt that the light absorbed by phycocyanin is used for photosynthesis with an efficiency approximating that of the light absorbed by chlorophyll. But whether the phycocyanin acts by transferring the energy it absorbs to chlorophyll, which then carries on photosynthesis in the same way as if it had absorbed the light initially, or whether the phycocyanin can carry out carbon dioxide assimilation independently of chlorophyll, is a question to which our experiments give no answer.

#### SUMMARY

The absorption spectra of the principal pigment components extracted from *Chroococcus* cells have been measured, and their sum compared with the absorption of a suspension of living cells. The agreement was sufficiently close so that it was concluded the absorption spectra of the extracted and separated pigment components could be used to obtain estimates of the relative absorption of the various components in the living cells.

The quantum yield of *Chroococcus* photosynthesis was measured at a succession of wave lengths throughout the visible spectrum, and the dependence of yield on wave length was compared with the proportions of light absorbed by the pigment components. This comparison showed beyond reasonable doubt that the light absorbed by phycocyanin is utilized in photosynthesis with an efficiency approximately equal to that of the light absorbed by chlorophyll. The light absorbed by the carotenoid pigments of *Chroococcus* seems for the most part to be unavailable for photosynthesis. The results leave open the possibility that light absorbed by the carotenoids is active in photosynthesis, but with an efficiency considerably lower than that of chlorophyll and phycocyanin. It is also possible that the light absorbed by one or a few of the several carotenoid components is utilized with a high efficiency, while the light absorbed by most of the components is lost for photosynthesis.

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# THE PHYSIOLOGICAL EFFECTS OF *L*-TYROSINE AND *L*-PHENYL-ALANINE ON THE RATE AND QUALITY OF PULSATION IN THE SEVENTY-TWO HOUR EXTIRPATED EMBRYONIC CHICK HEART\*

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The growth effects of the differentiation-enhancing amino acids, *L*-tyrosine and *L*-phenylalanine, have been reported in a previous communication.<sup>1</sup> The physiological effects upon rhythmicity and contractility of the 62, 72, and 96 hour chick hearts are here reported.

Since the 62 to 96 hour embryo chick heart is free of nervous control, the peculiar qualities of rhythmicity and contractility may be rightly attributed to either inherent potency, chemical influences, or both. By chemical influences is here meant either direct stimulation or depression due to naturally occurring compounds or to metabolic products of such substances upon the myocardial syncytium.

*L*-Tyrosine and *L*-phenylalanine bear structural relationship to epinephrine, ephedrine, tyramine, and thyroxine. These four products produce specific physiological manifestations which may easily be identified as such.

Consequently, the work reported in this paper was designed to determine the ease with which syncytial heart muscle of the 72 hour chick embryo could convert or utilize *L*-tyrosine or *L*-phenylalanine in this way. In addition, it was of interest, in the event that these naturally occurring amino acids would have any such action, to study the effects upon the quality of the pulsations and any alteration in the conduction pattern as compared to the known effects produced by *L*-epinephrine. Since these amino acids are present in such high concentrations in the hen's egg, it was thought that this information might have important bearing in relation to the initiation of rhythmicity in such hearts.

## *Method*

Fertile hen's eggs were obtained from a reputable hatchery within 24 hours after laying. These were incubated in a moist chamber incubator.

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\* Aided by a grant from Mrs. Elizabeth Nax.

<sup>1</sup> In press.

Locke's solution minus dextrose served as the basic medium. The electrolytic balance of Locke's solution is ideal for preserving the rhythmical pulsations of 72 hour chick hearts (1). Fletcher and Waters (2) report that mammalian heart muscle does not utilize dextrose when perfused with an artificial medium. The solution was prepared in triple distilled water, which had previously been boiled to remove excess carbon dioxide gas, and placed in a paraffin-lined bottle to prevent the silica of the glass from entering the solution.

In two experiments, the amount of *l*-tyrosine sufficient to make a  $m/100$  solution was dissolved in 1 cc. of a  $N/1$  HCl. Next, 8 cc. of a Locke's solution were added, and the solution finally brought to the original pH by the addition of 1 cc. of a  $N/1$  NaOH. The  $m/1,000$ ,  $m/10,000$ , and  $m/50,000$  dilutions of *l*-tyrosine were then prepared from the standard  $m/100$  solution. In the remaining experiments, sufficient *l*-tyrosine, *l*-phenylalanine, or *l*-epinephrine was dissolved in 100 cc. of Locke's solution to make the proper concentration. This latter method avoided even the slightest disturbance in the pH or osmotic pressure. In all cases the constituents<sup>2</sup> were weighed to the tenth part of a milligram on a high sensitivity analytical balance.

The blastoderm was excised, care being taken not to touch the heart or adjacent structures, and placed in 10 cc. of freshly prepared Locke's solution. The heart was then oriented under a dissecting microscope. The ectoderm was carefully removed from around the heart with an iridectomy knife. By means of a quick incision the sinus venosus was cut from the ducts of Cuvier, care being taken not to injure the sinoatrial node. The bulbus conus arteriosus was likewise divided from the aorta. The heart was then transferred to a large Petri dish containing 20 cc. of a freshly prepared Locke's solution by means of a wide mouth pipette. All experiments were conducted at 26°C.

The pulsation rate was taken soon after the heart was placed in the Locke's solution. Additional counts were taken every 15 minutes to a half hour until the last two rates agreed with each other within a variation of five pulsations per minute. As a rule, the faster beating hearts were transferred to the control dishes which contained 5 cc. of the normal Locke's solution, and slower hearts transferred to similar dishes containing the same amount of experimental solution in order that the increase in pulsation rate would exceed the refractory phase.

Pulsation rates were recorded for all hearts at half hour intervals. All experiments were concluded when experimental hearts had passed from irregular rhythm to partial or complete heart block rhythm.

The result of each experiment was plotted. At the conclusion of the study, all data were statistically combined by the method of graphic addition, and a single composite graph was made for each compound and concentration studied. Thirty-eight experimental 72 hour hearts and ten 72 hour control hearts were fixed in absolute alcohol, embedded in paraffin, sectioned, and stained for glycogen by the method of Best (3).

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<sup>2</sup> Amino acids were 99.8 per cent pure and were obtained from La Roche. Crystalline epinephrine was obtained from Armour and Co.

## EXPERIMENTAL OBSERVATIONS

*Control Hearts*

All individual curves show the same general form (Fig. 1). The composite rate curve statistically derived from the experimental data of fourteen control hearts revealed two peaks, the first of which occurred within 15 minutes after transfer from the original Locke's solution in which hearts remained until they assumed a regular pulsation rate, and a second peak which attained its greatest magnitude within 1 hour and 50 minutes after transfer to experimental

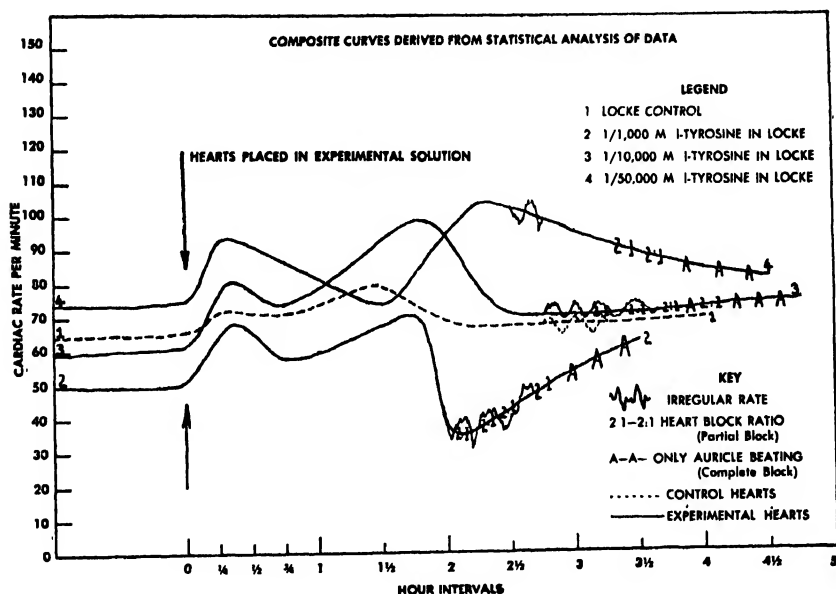


FIG. 1

control dishes of Locke's solution. The descending limb of the second peak continues to a lower level than that of the first peak. This continues at a comparatively uniform rate to the termination of the experiment. Individual control curves show increasing deterioration and decreasing pulsation rate beyond 4 hours and may fall to a level as low as eight to ten pulsations per minute.

35 per cent of control hearts do show a period of irregular rate manifest by the appearance of occasional pauses in pulsation between  $2\frac{3}{4}$  and  $3\frac{1}{4}$  hours. Control hearts did not assume a heart block rhythm but constantly pulsated at a normal rhythm with each auricular contraction followed by a contraction of the ventricle.



*Effect of l-Tyrosine*

The curves derived from the data of thirty-four experimental 72 hour chick hearts studied in  $M/1,000$ ,  $M/10,000$ , and  $M/50,000$  *l*-tyrosine showed the same fundamental features of the composite control curve with the following modifications (Fig. 1):—

1. There is an abrupt increase in pulsation rate in  $M/1000$ ,  $M/10,000$ , and  $M/50,000$  *l*-tyrosine, attaining a higher level than that seen in the composite control curve. The rate of pulsation was highest in  $M/10,000$  and  $M/50,000$  *l*-tyrosine.

2. In controls the succeeding decrease in pulsation rate is negligible. In the case of experimental solutions the following decrease in rate is more rapid. In  $M/1000$  *l*-tyrosine it is more rapid than in  $M/10,000$  and  $M/50,000$  *l*-tyrosine. However, the greatest fall in rate occurred in  $M/50,000$  solution.

3. The second period of increased pulsation rate in the experimental curve is relatively higher than that seen in the control curve. The pulsation rate is highest in  $M/10,000$  and  $M/50,000$  *l*-tyrosine, while with  $M/1000$  *l*-tyrosine the highest pulsation rate is slightly above that seen soon after transfer to *l*-tyrosine solutions.

In all cases this highest pulsation rate is delayed in comparison with the controls, appearing earliest in  $M/1000$  *l*-tyrosine and latest in  $M/50,000$  *l*-tyrosine.

4. The degree of pulsation depression decreases with increasing concentration of *l*-tyrosine. In all cases the depression of rate is greater than that of controls.

5. Recovery from the previous depression is more rapid with decreasing concentration of *l*-tyrosine.

6. Irregularity of rhythm appears first in experimental conditions. Irregularity is seen at lowest depression in the case of  $M/1000$  *l*-tyrosine and during beginning depression in  $M/50,000$  *l*-tyrosine, while irregularity is seen during recovery in  $M/10,000$  *l*-tyrosine.

7. Irregularity is soon replaced by partial or complete heart block rhythm in 75 per cent of experimental hearts. In the case of  $M/50,000$  *l*-tyrosine, heart block rhythm is established during depression while in the case of  $M/1000$  and  $M/10,000$  *l*-tyrosine heart block rhythm is established during recovery from the previous depression. The usual rhythm observed is 2 to 1, but 3 to 2, and 10 to 1 rhythm have been occasionally observed.

8. In all cases the terminal pulsation rates of experimental hearts are relatively higher than the terminal pulsation rates of control hearts.

When placed in fresh Locke's solution, only 20 per cent of 72 hour experimental hearts show recovery of rate and rhythm.

Four 64 hour embryo chick hearts were only slightly susceptible to the effects

of *L*-tyrosine. These showed immediate depression in  $M/1,000$  *L*-tyrosine solution, and only the slightest increase in pulsation rate. There was, however, some stimulation followed by a greater depression and irregular rhythm in  $M/10,000$  *L*-tyrosine, but in no case did the effect compare with that of 72 hour embryo chick hearts in *L*-tyrosine solution.

*L*-Tyrosine exerted its greatest effect on 96 hour embryo chick hearts. Four 96 hour hearts placed in  $M/1,000$  solutions showed an immediate and sustained increase of pulsation rate. Pulsation irregularity sets in at peak stimulation and was immediately followed by partial heart block rhythm.

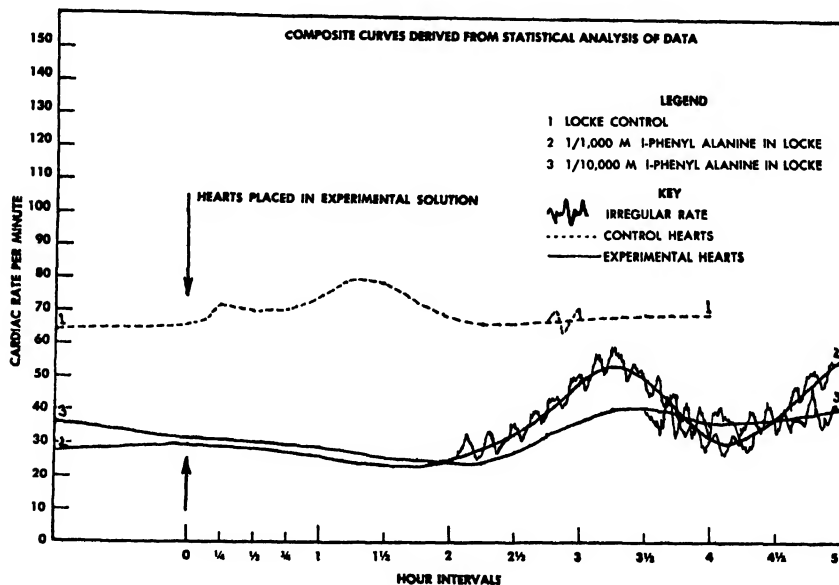


FIG. 2

On the other hand  $M/10,000$  *L*-tyrosine produced depression of pulsation rate after peak stimulation followed by a gradual recovery, pulsation irregularity, and partial heart block rhythm, or else complete cessation of beat. All hearts showed complete recovery of rhythm and pulsation rate when placed back into the normal Locke's solution.

It was found that *L*-epinephrine had no consistent effect, especially in concentrations below  $M/10,000$ . Ten hearts were observed in *L*-epinephrine. Some experimental hearts did show an immediate stimulation occurring within 15 minutes after transfer to the experimental solution. This was followed by depression and the appearance of partial heart block rhythm. A second stimulation peak was observed only in  $M/100,000$  *L*-epinephrine, but in this case there was also irregularity of rhythm. It is to be emphasized

that any results with *l*-epinephrine are to be cautiously evaluated since the compound is rapidly oxidized in aqueous solution.

### *Effects of Phenylalanine*

The curves derived from the data of ten experimental 72 hour chick hearts studied in  $M/1000$  and  $M/10,000$  *l*-phenylalanine show the same fundamental features of the control curve with the following modifications (Fig. 2):—

1. There is a gradual and prolonged depression of pulsation rate immediately upon transfer to  $M/1000$  and  $M/10,000$  *l*-phenylalanine. Depression is greater in  $M/10,000$  *l*-phenylalanine.

2. Highest pulsation rate in  $M/1000$  *l*-phenylalanine is greatly delayed in relation to the control curve, but eventually it attains a magnitude equal to that seen in  $M/50,000$  *l*-tyrosine. The highest stimulation in  $M/10,000$  is delayed beyond that of  $M/1000$  and is of lesser magnitude.

3. The following depression of rate is most severe and rapid in  $M/1000$ . This is followed by an increasing pulsation rate which attains a level equal to the previous peak value.

4. In the case of  $M/1000$  *l*-phenylalanine the first increase in pulsation rate is accompanied by the appearance of persisting irregularity. In  $M/10,000$  *l*-phenyl-alanine irregularity appears at the peak pulsation rate.

### DISCUSSION

The first peak in the composite control curve is apparently the result of mechanical stimulation since this does not occur if hearts are not transferred. If this peak be graphically eliminated by the string method, it will be seen that the pulsation rate gradually increases from the beginning; at 45 minutes the increase in rate becomes more rapid, and the rate attains its greatest magnitude in 1 hour and 45 minutes. This latter, sudden increase in rate is not surprising since the nodal tissues are markedly sensitive to  $CO_2$ , which causes an increased rate of impulse initiation at the sino-atrial node and increased conduction over the arterio-ventricular bundle in fully developed hearts. The rapid fall of pulsation rate below the base line may be attributed to the overwhelming effects of  $CO_2$  and other metabolites which cause slowing of the heart and increased tone of the cardio-inhibitor center of hearts subject to nervous control. Beyond the 3 hour interval, individual control pulsation curves show steadily increasing depression.

There is evidence of irregularity in 35 per cent of control hearts. This may be attributed to the accumulation of carbon dioxide, since it was noted that a fall in pH to a level of 7 of Locke's solution causes extra systoles, heart block, other irregularities, followed by a cessation of impulse initiation and conduction in 72 hour embryo chick hearts.

Hearts placed in *l*-tyrosine show an initial stimulation followed by a de-

pression. With  $m/1000$  *l*-tyrosine the depression predominates whereas with  $m/10,000$  and  $m/50,000$  *l*-tyrosine stimulation was more evident. Stimulation was manifest as an increase in rate and conduction and depression as a decrease in rate and conduction. The period of irregularity appears to approach the peak stimulation as the concentration of *l*-tyrosine is decreased. Irregularity of rhythm and associated heart block rhythms appear to be true conduction effects and result from the direct injury suffered by the nodal tissue while acceleration of pulsation, due to increased impulse initiation is apparently the result of a direct effect on the nodal tissues.

These effects closely resemble the effects of *l*-epinephrine on the intact mammalian heart (Sollmann, 4).

The results of experiments with 62 hour, 72 hour, and 96 hour chick hearts indicate that the degree of sensitivity to *l*-tyrosine parallels differentiation of the tissue.

Adrenalin is first detected, by present available methods, on the 8th day of embryonic development (5). 5-day embryo chick hearts have been shown to be sensitive to epinephrine (6). We have found that 72 hour chick hearts give varied responses to 1 part in 10 thousand, and 1 part in 50,000 crystalline *l*-epinephrine.

*l*-Phenylalanine produces an initial depression; later it has effects similar to those of *l*-tyrosine.  $m/10,000$  *l*-phenylalanine is toxic and causes a steadily increasing depression immediately after transfer followed by the typical *l*-tyrosine effect, which in the case of  $m/1000$  attains a level twice as high as that observed with  $m/10,000$  *l*-phenylalanine. The period of irregularity differs from that produced by *l*-tyrosine. In this case irregularity is seen at lowest depression and not peak stimulation. There is no recovery of hearts when placed in fresh Locke's solution such as occurred after tyrosine. Instead hearts go into partial or complete heart block.

*l*-Phenylalanine is an indispensable amino acid (7), and may serve as the immediate precursor of *l*-tyrosine, which in turn is readily converted to *l*-epinephrine, as has been shown in kidney slices (8). The initial depression possibly represents immediate toxicity and lag before the compound is converted to *l*-tyrosine. The initial toxicity may also be due to the conversion of part of the *l*-phenylalanine into *l*-phenyl pyruvic acid (9), which is not easily oxidized by tissues. In this case the stimulation level would be lower than that seen with the same concentration of *l*-tyrosine because of the presence of an inhibiting compound or initial injury suffered by the myocardial syncytium.

The embryo chick heart first begins to pulsate rhythmically after 36 hours. Ganglion cells cannot be demonstrated as such until 148 hours. The sympathetic nervous system does not appear until after 120 hours. This indicates that the peculiar qualities of rhythmicity and contractility are inherent and

myogenic. If one attempts to go a step further, he may deduce that other inherent qualities and potencies are likewise dependent upon non-nervous factors—probably chemical in nature. It is well established that the mineral constituents of the blood such as Ca, K, Na, O<sub>2</sub>, and CO<sub>2</sub> tensions, lactic acid, and *l*-epinephrine exert important influences on the heart, either directly or indirectly by their actions upon the hypothetical substance of Langely. According to Cruickshank and McClure (10), the amino acids are very sparingly utilized as a source of energy. The combustion of fat serves as the greatest energy source. The amino acids, however, have been reputed to be of greater significance in cardiac metabolism since they exert their effects through certain intermediaries and metabolites such as tyramine and epinephrine and so condition the inherent qualities of heart muscle. This of course is opposed to the belief that *l*-epinephrine is a metabolic waste. Mitchell and Hamilton (11) report the presence of 4 per cent *l*-tyrosine and 5.1 per cent *l*-phenylalanine in egg albumin. Since *l*-phenylalanine is considered the immediate precursor of *l*-tyrosine, the initial concentration of *l*-phenylalanine was probably higher. Galvialo (12) reports the existence of a protease, amylase, and lipase in the 24 hour chick embryo, while the presence of a tyrosinase, and catalase, have been reported in the yolk of Koga (13). This indicates that the facilities for the conversion of *l*-phenylalanine or *l*-tyrosine into *l*-epinephrine and tyramine are present before any recognizable rudiment of the adrenal glands appear and before *l*-epinephrine can be pharmacologically detected. Possibly *l*-tyrosine or *l*-phenylalanine is concerned with the initiation and preservation of rhythmicity in the heart.

Thanks are extended to Drs. Stanley P. Reimann and J. Earl Thomas for their assistance during the course of this work and preparation of the manuscript.

#### SUMMARY

1. 72 hour isolated chick hearts show an increase in pulsation rate when placed in  $m/1000$ ,  $m/10,000$ , and  $m/50,000$  *l*-tyrosine solutions. The optimal effect is seen in  $m/10,000$  and  $m/50,000$  *l*-tyrosine.

2. All hearts show disturbance of rhythm either in the form of irregular rhythm or heart block.

3. 62 hour isolated chick hearts are not susceptible to *l*-tyrosine while 96 hour hearts are markedly sensitive.

4. 72 hour isolated chick hearts placed in 1 part in 10,000 and 1 part in 50,000 *l*-epinephrine show approximately the same effects as were seen with *l*-tyrosine.

5. 72 hour isolated chick hearts placed in  $m/1000$  and  $m/10,000$  *l*-phenylalanine show an initial depression followed by an *l*-tyrosine effect.

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# THE FLOCCULATION MAXIMUM (pH) OF FIBRINOGEN AND SOME OTHER BLOOD-CLOTTING REAGENTS. (RELATIVE TURBIDIMETRY WITH THE EVELYN PHOTOELECTRIC COLORIMETER)

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In previous publications (4, 5, 12) it has been pointed out that *fibrinogen*, as ordinarily prepared from plasma by "salting-out" with neutral salts, is not a pure protein, but contains significant amounts of contaminants, including serum globulin, prothrombin, phospholipids, inorganic salts, etc. It is a common experience that fibrinogen solutions keep poorly and cannot be evaporated without irreversible change (denaturation). A successful "lyophile" preparation has been described, however (8). The deterioration which occurs in solutions and sometimes even during the course of preparation (particularly if too many "saltings" are attempted) is usually regarded as a form of *denaturation*, but there are indubitable occasions when some true *fibrin clot* appears, despite all the usual precautions. By means of cold-room temperature (2°C.) and moderate acidification (pH = 6.0), Florkin (11) obtained a NaCl fibrinogen which fulfilled the physicochemical requirements of constant and reproducible solubility, held to permit its characterization as "sufficiently purified of other proteins and its own denaturation products to yield a chemical individual." Nevertheless, the preparation was very unstable at room temperature, especially at alkaline pH, and it could not be dialyzed. May not Florkin's limited success be attributable to the known ability of cold and acidity to arrest the action of a contaminating trypsin-like enzyme?

Such an enzyme (serum *tryptase*) is well recognized and numerous experiments in the past few years (2, 3, 9, 12, 15) attest its importance (as a constituent both of plasma and of aqueous tissue extracts, or "thromboplastin") in the mechanisms of blood coagulation (6). Both crude tissue (and plasma) materials and the essentially analogous pure crystalline trypsin (isolated from pancreas by Northrop and Kunitz in 1936) show parallelism of behavior, under appropriate experimental conditions, with respect to (a) fibrinogenolysis and fibrinolysis (2, 9), (b) prothrombinolysis (7) and thrombinolysis (= *progressive* antithrombin (12)), (c) thromboplastic acceleration of the conversion of prothrombin into thrombin (2) particularly in the presence of optimal amounts of



$\text{Ca}^{++}$  and cephalin (P lipid) (9), (d) inhibition by heparin (13, 10). Trypsin is unable to clot fibrinogen directly but only through facilitating thrombin production (2, 9, 15), and this facilitation can proceed under conditions of great depression of *Ca* ionization (2, cf. 9), which argues strongly for the probability that the natural plasma tryptase, in addition to proteolytic effects, may produce at least traces of true fibrin in kept fibrinogen solutions containing small amounts of prothrombin. It then becomes a question whether any of the numerous experiments, which are interpreted in the coagulation literature as fibrinogen "denaturation," are free from confusion with true fibrin clotting arising in this way.

The extensive work of Wöhlisch and colleagues (excellently summarized in a recent review (15)) is an impressive correlation of denaturation and coagulation phenomena in an endeavor to support Wöhlisch's theory that the fibrin clotting of fibrinogen is merely a type of denaturation, specifically catalyzed by a unique proteolytic enzyme, namely, thrombin, not to mention papain and certain venoms (cf. 2, 3, 6). Conservative opinion emphasizes, rather than minimizes, the experimental differences between fibrin and denatured fibrinogen. The new data on tryptase activities also undermine much of the evidence, such as it is, for a protease-like character of thrombin.

Certain data advanced by Wöhlisch and others (15) deal with the *flocculation maximum* of fibrinogen in buffer solutions of varying pH and salt content. It may be regarded as established that (a) fibrinogen regularly shows a flocculation optimum well on the acid side of neutrality, the exact location of which is determined by the electrolyte concentration, (b) the optimum is independent of the protein concentration, but this, in conjunction with the salt content, determines the "range" of precipitability. The questionable datum concerns an alleged "second maximum" to the alkaline side of the "chief" maximum. Such an appearance is well known in the denaturation of egg albumin and caseinogen, and Wöhlisch argues for a similar phenomenon in the case of fibrinogen denaturation. The real issue, however, arises over claims for a comparison with fibrin. Owing to its peculiar *quasi*-crystalline (oriented micelle) gel formation, *fibrin* does not lend itself to flocculation experiments (*v. infra*), and arguments as to its "maximum of precipitability" in a pH region closer to the neutral point, are indirect and somewhat lacking in conviction. Later workers, whom Wöhlisch (15) quotes in confirmation, have improved upon that author's original methods by introducing turbidimetric techniques, including the use of the Zeiss-Pulfrich *Stufenphotometer*.

Having at hand a limited quantity of a particularly suitable fibrinogen preparation (stable and prothrombin-free, *v. infra*), together with the excellent instrumentation of the Evelyn *photoelectric colorimeter*, we have sought to re-investigate the particular problem outlined above.

### Methods

**Reagents.**—1. *Plasma*.—Obtained from citrated (0.38 per cent) dog blood by two centrifugalizations and passing *rapidly* through a Berkefeld V ultrafilter. It was 7 to 10 days old at the time of the cited tests but appeared stable and yielded a clear solution on routine filtration to get rid of a negligible trace of flocculent deposit.

2. *Fibrinogen*.—Precipitated three times from the above plasma (24 hours old) by one-fifth saturation with  $(\text{NH}_4)_2\text{SO}_4$ . The final preparation, dissolved in 0.85 per cent NaCl at pH = 7.0, was a clear opalescent solution, stable for more than a week in the ice box at 5°C., despite frequent exposures for hours at room temperatures (25°C.). Throughout the period of study there was no trace of sediment or clotting of any kind. Ca salt, *with* and without added thromboplastin (weak saline extract of frozen dog brain), gave no clot during 48 hours, thus proving the complete absence of all traces of prothrombin. Solid clots were obtained in a matter of seconds with a purified thrombin solution (5) and there was no fibrinolysis in 3 days. The fibrin yield, obtained gravimetrically after such thrombin clotting, amounted to 0.375 gm. per 100 cc. solution.

3. *Preheated Fibrinogen*.—10 cc. fibrinogen carefully warmed, in water bath, until incipient turbidization (at 51°C.), then cooled immediately (cold-tap), to prevent flocculation.

4. *Acetate Buffer Solutions*.—Made from N/50 acetic acid and N/50 potassium acetate, were checked with the glass electrode (Beckmann pH meter).

5. *Trypsin*.—1:1000 crystalline trypsin (Plaut Research Laboratory), in distilled water. The quantities cited in the tests refer to dry weight of the enzyme preparation (which includes about 60 per cent  $\text{MgSO}_4$ ) per unit volume of protein solution tested.

**Apparatus and Technique.**—The Evelyn photoelectric colorimeter was adjusted for 10 cc. (macro) samples, using a green filter (540) and matched tubes (blank =  $82.0 \pm 1.0$ ). 9.5 cc. of buffer solution were placed in tube and adjusted to give a zero (+ 100.0) position of the galvanometer spot-light. 0.5 cc. of protein solution was added, mixed, and readings taken (as routine) at 15 seconds, and 1, 2, 5 minutes. In Experiments III and VII 1/10 volume of protein solution was used, but a slight tendency to *late* flocculation (with resulting reversal of reading) inclined us to prefer the 1/20 volume above suggested. The relative turbidities, as measured by the galvanometer scale deflection, are given for the 1 minute readings, in the data summarized in Table I. There was usually a fairly definite maximum within the 5 minute observation period, but the slight tendency to drift (partly inherent in the instrument) made it undesirable to attempt to fix the "maxima," particularly since the later readings invariably supported the sequence recorded for the 1 minute series. While 1.0 scale unit is probably significant, we have arbitrarily adopted a minimum of 2.0 units, over the initial turbidity, to define the somewhat indefinite limits of the "range" of turbidization. This gives results, for the 1 minute series, within the range of allowed error of 0.2 pH units of buffer variation (*v. infra*).

**Data (Table I).**—

**A. Stable Fibrinogen.**—I. 1/20 volume in N/100 acetate buffer: flocculation maximum (F.M.), pH = 5.3 ( $\pm 0.2$ ); range = 5.9 — 4.3 ( $\pm 0.2$ ). II. 1/20 volume

in N/100 acetate buffer, containing 1 per cent NaCl, F.M. = 4.7 ( $\pm 0.2$ ); range = 5.1 - 4.1 ( $\pm 0.2$ ). III. 1/10 volume in N/50 acetate buffer: F.M. = 4.7 ( $\pm 0.2$ ); range = 5.3 - 3.9 ( $\pm 0.2$ ).

TABLE I

*Flocculation Maximum of Fibrinogen and Plasma in Acetate Buffers of Varying Salt Content. Relative Turbidimetry with Evelyn Photoelectric Colorimeter*

No.	Initial pH	Checked pH (V)	Various fibrinogen preparations							Plasma			
			I	II	III	IV	V	VI	VII	VIII	IX	X	XI
1	6.47	6.43	0.5	0.5	1.9	4.0	2.2	2.0	1.5	1.0	1.5	6.0	3.0
2	6.3	6.25	0.8	0.4	2.0	5.1	2.0	2.0	1.4	1.2	6.0	8.0	8.0
3	6.1	6.10	1.4	0.2	1.9	6.8	3.0	1.5	2.0	1.5	8.8	9.0	9.5
4	5.9	5.89	7.7	0.8	1.9	11.5	2.8	1.8	0.5	1.1	18.1	19.0	17.0
5	5.7	5.70	16.0	0.8	2.0	16.5	5.0	1.2	0.9	1.5	30.0	32.0	27.5
6	5.5	5.46	23.5	0.5	6.5	22.5	5.0	2.5	1.0	1.0	47.0	44.0	38.5
7	5.3	5.26	24.4	0.5	24.0	24.0	6.5	3.0	1.4	1.1	57.0	53.0	50.0
8	5.1	5.02	23.5	3.5	39.0	23.7	14.0	9.0	1.9	2.5	55.5	53.0	50.0
9	4.9	4.81	18.5	18.8	47.0	20.7	16.8	16.0	9.5	13.5	35.0	41.0	35.0
10	4.7	4.61	16.2	27.0	53.0	17.5	18.0	23.5	19.0	15.0	31.0	35.5	31.5
11	4.5	4.42	13.0	25.5	50.0	14.0	15.5	23.0	21.0	13.0	25.0	31.0	27.0
12	4.3	4.24	6.0	22.0	39.0	8.5	11.0	22.0	17.0	9.0	20.0	25.0	24.0
13	4.1	4.05	1.0	14.5	28.0	3.0	3.5	12.0	10.4	1.5	13.0	12.0	18.0
14	3.9	3.87	0.5	1.8	6.0	1.0	2.0	3.8	++	1.1	6.0	4.5	12.5
15	3.7	3.68	0.2	0.8	2.0	0.5	1.0	2.4	++	0.8	4.0	3.0	14.0

I. 0.5 cc. stable fibrinogen +9.5 cc. acetate buffer (N/100).

II. 0.5 cc. " " +9.5 cc. " " (N/100), containing 1 per cent NaCl.

III. 1.0 cc. " " +9.0 cc. " " (N/50).

IV. 0.5 cc. predenatured (51°C.) fibrinogen +9.5 cc. acetate buffer (N/100).

V. 0.5 cc. " " +9.5 cc. " " (N/100) containing 0.5 per cent NaCl.

VI. 0.5 cc. predenatured (51°C.) fibrinogen +9.5 cc. acetate buffer (N/100) containing 1.0 per cent NaCl.

VII. 1.0 cc. tryptic digest of fibrinogen +9.5 cc. acetate buffer (N/50).

VIII. 0.5 cc. plasma +9.5 cc. acetate buffer (N/100), containing 0.25 per cent NaCl.

IX. 0.5 cc. " +9.5 cc. " " (N/100).

X. 0.5 cc. plasma, preheated (53°C.) +9.5 cc. acetate buffer (N/100).

XI. 0.5 cc. plasma, digested for 1 hr. with trypsin (1:100,000) +9.5 cc. acetate buffer (N/100).

*B. Preheated (51°C.) Fibrinogen.*—IV. 1/20 volume in N/100 acetate buffer: F.M. = 5.3 ( $\pm 0.2$ ); range = >6.45 - 4.1 ( $\pm 0.2$ ). V. 1/20 volume in N/100 acetate buffer, containing 0.5 per cent NaCl: F.M. = 4.7 ( $\pm 0.2$ ); ranges = 5.7 - 4.1 ( $\pm 0.2$ ). VI. 1/20 volume in N/100 acetate buffer, containing 1 per cent NaCl: F.M. = 4.7 ( $\pm 0.2$ ); range = 5.5 - 3.7 ( $\pm 0.2$ ).

*C. Tryptic Digest of Fibrinogen.*—VII. 1/10 volume of fibrinogen (digested for 1 hour with 1:10,000 trypsin) in N/50 acetate buffer: F.M. = 4.5 ( $\pm 0.2$ ); range = 4.9 - <3.7.

*D. Plasma.*—VIII. 1/20 volume in N/100 acetate buffer, containing 0.25 per cent NaCl: F.M. = 4.7 ( $\pm 0.2$ ); range = 5.1 – 4.3 ( $\pm 0.2$ ). IX. 1/20 volume in N/100 acetate buffer: F.M. = 5.3 ( $\pm 0.2$ ), range = 6.3 – <3.7. X. 1/20 volume of preheated (53°C. = incipient turbidity) plasma in N/100 acetate buffer: F.M. = 5.2 ( $\pm 0.4$ ), range = 6.1 – 4.3. XI. 1/20 volume of plasma (digested for 1 hour with 1:100,000 trypsin) in N/100 acetate buffer: F.M. = 5.2 ( $\pm 0.4$ ), range = 6.3 – <3.7.

#### DISCUSSION OF DATA

The pH check made on the final mixtures, in the case of series V, is quite satisfactory. At the most, there might be a correction of  $-0.1$  pH, but this is well within the allowed limit of  $0.2$  pH, represented by the series, and the slight correction may be neglected.

The stable fibrinogen shows a F.M. of pH = 5.3 ( $\pm 0.2$ ) in electrolyte-poor buffer, but a prompt and decided shift to the acid (pH = 4.7,  $\pm 0.2$ ) on doubling the buffer strength or adding 1 per cent NaCl.

Preheating to 51°C. (incipient denaturation), gave fibrinogen preparations which displayed *exactly the same* F.M. as the untreated fibrinogen, *viz.* pH = 5.3 ( $\pm 0.2$ ) in salt-poor buffer, and pH = 4.7 ( $\pm 0.2$ ) in N/100 acetate containing 0.5 or 1.0 per cent NaCl. There was no appearance of any "second maximum," although the experimental conditions with respect to buffer and salt concentrations closely duplicate those under which Wöhlich (15) claimed to show such appearance in crude fibrinogen preparations.

There was only enough material available for one experiment with trypsin. 1/10 volume of 1:1000 crystalline trypsin, acting for an hour or two, rendered the fibrinogen solution incoagulable by a thrombin, which gave solid clots in a fraction of a minute with the untreated and the preheated fibrinogen. Trypsin has obviously produced fibrinogenolysis, but there is sufficient precipitable protein left in the clear mixture to give a marked turbidity in ten volumes of N/50 acetate buffer. The lessened opacity of VII as compared with III, and also the somewhat narrower range of turbidization, suggest a diminution in total protein content. The F.M. at pH = 4.5 ( $\pm 0.2$ ) is not significantly different from that of the other fibrinogens.

The experiments with the Berkefeld-filtered plasma, from which the fibrinogen had been prepared, and also submitted to partial heat denaturation and tryptic digestion, again indicate a single flocculation maximum at pH = 5.3 ( $\pm 0.2$ ) in electrolyte-poor buffer and the usual shift to pH = 4.7 ( $\pm 0.2$ ) in N/100 acetate containing 0.25 per cent NaCl. The turbidization technique is obviously unable to distinguish between the various plasma protein fractions.

On adding 1/20 volume N/10  $\text{CaCl}_2$  to the 10 day old citrated plasma (an amount insufficient to produce coagulation), there was no change in the precipitation pattern (hence data omitted from Table I). On adding 1/5 volume of N/10  $\text{CaCl}_2$ , clotting occurred in 15 minutes with prompt reversal

of turbidity in the tube (pH = 5.9) reached in the flocculation study. The solidification of the plasma, of course, prevented continuation of the experiment. There was an interesting fluctuation of the galvanometer deflection in the zone about the F.M. or clot occurrence, in both these experiments. This is probably due to light dispersion by the sparse *fibrin* filaments formed.

*Coagulation Data.*—Experiments X and XI were performed on plasma 9 days old. The following *clotting tests* were run at 21.5°C., pH = 8.0.

1. 1.0 cc. plasma + 0.25 cc. N/10  $\text{CaCl}_2$  = solid clot in 8½ minutes.
2. 1.0 cc. plasma + 0.25 cc. N/10  $\text{CaCl}_2$  + 0.1 cc. brain thromboplastin = solid clot in 25 seconds.
3. 1.0 cc. plasma + 0.25 cc. N/10  $\text{CaCl}_2$  + 1:100,000 trypsin = solid clot in 3 minutes.
4. 1.0 cc. plasma + 1:100,000 trypsin (as in Experiment XI) = no clot in 24 hours; trace on 3rd day.
5. 1.0 cc. plasma + 1:100,000 trypsin (as in Experiment XI), recalcified (0.25 cc. N/10  $\text{CaCl}_2$ ) after 2 hours (*i.e.* at conclusion of Experiment XI) = solid clot in 11 minutes.

These tests permit the following conclusions:

1. The plasma (9 days old) shows excellent preservation of its clotting properties, including abundance of fibrinogen, prothrombin, and some thromboplastin (since Ca salt alone is sufficient to induce coagulation).
2. The amount of trypsin used in Experiment XI is insufficient to cause any fibrin clotting in the citrated plasma in the absence of added calcium.
3. Nevertheless, the longer time (11 minutes) in test 5, as compared with clotting time of 3 minutes in test 3 (identical, except for the duration of exposure to 1:100,000 trypsin), is good evidence that trypsin has exerted considerable digestive action on the clotting agents (prothrombin and fibrinogen).

Thus, Experiment XI (Table I) clearly deals with a protein mixture, subjected to some tryptic digestion, without the occurrence of fibrin formation. The extension of the flocculation range in both XI and VII points to an effect of tryptic digestion on the precipitability of proteins in the acid region (pH below 4.0), which is not adequately studied with the buffer range employed, and which is obviously irrelevant to the question of a "second maximum" near neutrality, namely, the problem under investigation.

#### COMMENT

The present material and methods offer an exceptional opportunity to secure much needed new data on alleged changes in the flocculation behavior of *fibrinogen* in buffered solutions of varying salt content. The failure to alter the precipitation pattern by mild thermal denaturation contradicts the findings made by earlier observers with less suitable materials, and questions the validity of analogies to denaturing ovalbumin and caseinogen. Fibrinogen which

has been lysed by crystalline trypsin (to loss of coagulability with thrombin) still shows the same precipitation behavior.

From the present results it can be definitely concluded that the procedures mentioned fail to cause the formation of any substance distinguishable by the flocculation technique and the earlier reports must, therefore, refer to material which is not a "genuine" constituent of native or denatured or digested true fibrogen. If Wöhlisch (15) is correct in drawing a parallelism between his "second maximum" substance and fibrin (or Apitz's alleged *profibrin*), this would seem to suggest that fibrin is actually formed under the experimental conditions with the crude preparations used. Knowing the ubiquitous presence in such materials not only of prothrombin but also of tryptase-like "thromboplastic enzyme," which is entirely capable of activating the prothrombin to thrombin and hence producing some conversion of fibrinogen to fibrin, this suggestion becomes an experimental contingency which must be ruled out before any conclusions can be drawn as to possible relationships between denaturation and coagulation. The same contingency, that actual fibrin formation may proceed *pari passu* with denaturation of fibrinogen, throws doubt upon many of the numerous experiments of the Würzburg school. The fact, for instance, that preheated fibrinogen clots more quickly with subsequently added thrombin, may be due merely to incipient coagulation by traces of thrombin impurity, the formation of which (from prothrombin) is accelerated by the warming. At times, this evinces itself in "spontaneous" clotting (indubitably fibrin), while on other occasions, because of the slow production of the traces of fibrin and subsequent fibrinolytic destruction by the same tryptase enzyme (upon which the whole process depends), the fibrin itself is missed and one merely notes the deterioration of the fibrinogen preparation and the eventual loss of its coagulability.

We believe that conservative judgment, based upon obvious physical differences, *e.g.* (a) appearance, gross and microscopic (especially dark-field), (b) solubility relationships, etc. (15), requires us to look upon fibrin formation as a *specific* phenomenon of the action upon fibrinogen of thrombin and a few unusual proteases (papain, certain venoms). It is *not* to be identified with non-specific processes of protein denaturation or digestion, although it is a matter of observation that all three processes may be simultaneously demonstrable in crude plasma and clotting systems prepared therefrom. It is very necessary that the biochemist and physical chemist have accurate knowledge of the factors involved in blood coagulation, and of the conditions under which they may act, in order to establish criteria of "purification" of the individual proteins and enzymes, etc. which may be involved. The present study attempts to give significance to these matters, and the chance obtaining of a fibrinogen apparently free from prothrombin and protease impurities, affords a unique opportunity to secure the cited data, which obviously fail to support the thesis

of an observable common alteration in the precipitation pattern for at least two of the three processes mentioned. Whether or not the entry of the third process (true fibrin clotting) can explain the "second maximum" described in the literature is not completely proved by the above results, although the otherwise unsatisfactory experiments with recalcified plasma do suggest it. It is clear that when fibrin formation can be ruled out, denaturation or digestion are unable to elicit the phenomenon in question.

Our flocculation maximum ( $\text{pH} = 4.7 \pm 0.2$ ) is in good agreement with the most reliable data in the literature (15). The value of  $\text{pH} = 5.3 \pm 0.2$ , in electrolyte-poor buffer, is close to the figure ( $\text{pH} = 5.4$ ) given by Stenhagen (14) for the "isoelectric point," by cataphoresis, in the Tiselius apparatus. In view of the evidence that we are working with an exceptionally reliable fibrinogen, it may be suggested that a pH of about 5.4 represents the flocculation maximum in the *salt-free* condition, such as presumably obtains also at the null point during electrophoresis, owing to the very great mobility of salt ions as compared with the slow moving protein micelles. Coincidence of the values obtained by two such different methods is presumptive evidence that we are dealing at this pH, with the *true isoelectric point* of fibrinogen protein proper.

#### *Other Uses of the Photoelectric Colorimeter in Coagulation Research*

Some studies, similar to the foregoing, have been made on our Howell type *prothrombin* solution (5), before and after activation to *thrombin*. In both cases, there was a single definite "flocculation maximum" at  $\text{pH} = 5.4 (\pm 0.2)$ , with the rather small turbidimetric value (*cf.* Table I) of 10.0 scale units. The turbidity "range," in the weak protein solutions (not assayed), was  $\text{pH} = 6.1\text{--}4.7 (\pm 0.2)$ , in both instances. The thrombic mixture consisted of 9 cc. prothrombin solution + 0.5 cc.  $\text{CaCl}_2$  (N/10) + 0.5 cc. of a very weak saline extract of frozen dog brain. This *thromboplastin* solution (filtered), tested independently, had an initial turbidity of 10 scale units, to which was added a low maximum of 5 units at  $\text{pH} = 4.7$ . In all these experiments, the mixture consisted of 0.5 cc. of crude protein + 9.5 cc. N/100 acetate buffer.

Baldes and Nygaard (1) have developed a "coagelometer" on principles which differ in no significant particular from the Evelyn apparatus, as used to measure relative turbidities in the manner above described.<sup>1</sup> By successive readings after the addition of thrombin to fibrinogen, the *kinetics of the second phase* of clotting (=fibrin formation) can be followed accurately, from the first turbidity appreciable to the photoelectric cell to the completion of the process, including late fibrinolysis (if any).

In some crude tests using plasma, instead of fibrinogen, we noted that the

<sup>1</sup> The addition of a photographic unit to obtain a continuous record of the galvanometer deflection is the one noteworthy difference in the Nygaard apparatus.

opacity took so long to develop a "maximum," that the quantitative measurements of relative turbidity are subject to a complex evaluation of the experimental conditions. However, *with a 1:10 saline dilution of plasma*, the onset of clotting is sharp and a definite "maximum" is reached within a minute or two. On adding serial quantities of the above stable fibrinogen solution to this diluted plasma and then clotting the mixture with thrombin, the maximum was found to increase significantly with as little as 1/50 volume of added fibrinogen solution. Under the cited conditions, therefore, it is possible to measure one-fiftieth of 0.375 per cent fibrinogen, *i.e.* 7.5 mg. in 100 cc., a remarkable degree of sensitivity for a protein assay. Without entering into a discussion of the applicability of such a technique, and the validity of the necessary *standards* of reference, it is obvious that the Evelyn instrument has a special field of usefulness in turbidimetric (nephelometric) assays of many kinds.

It is doubtful whether the turbidimetric *timing* of either onset or completion (solid gel) of fibrin clotting is significantly better than with simple visual means.

#### SUMMARY

By means of a novel adaptation of the Evelyn photoelectric colorimeter to the measurement of relative turbidities, the question of the *flocculation maximum* (F.M.) in acetate buffer solutions of varying pII and salt content has been studied on (a) an exceptionally stable prothrombin-free fibrinogen and its solutions after incipient thermal denaturation and incomplete tryptic proteolysis, (b) plasma, similarly treated, (c) prothrombin, thrombin, and (brain) thromboplastin solutions.

All the fibrinogens show a remarkable uniformity of the precipitation pattern, *viz.* F.M. = 4.7 ( $\pm 0.2$ ) pH in salt-containing buffer solutions and pH = 5.3 ( $\pm 0.2$ ) in salt-poor buffer (N/100 acetate). The latter approximates the *isoelectric point* (5.4) obtained by cataphoresis (14). There is no evidence that denaturation or digestion can produce any "second maximum." The data support the view that fibrin formation (under the specific influence of thrombin) is intrinsically unrelated to denaturation and digestion phenomena, although all three can proceed simultaneously in crude materials. A criticism is offered, therefore, of Wöhlisch's blood clotting theory.

Further applications of the photoelectric colorimeter to coagulation problems are suggested, including kinetic study of fibrin formation and the assay of fibrinogen, with a possible sensitivity of 7.5 mg. protein in 100 cc. solution.

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# THE PRESENCE OF AN ENDOGENOUS RESPIRATION IN THE AUTOTROPHIC BACTERIA

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*Thiobacillus thiooxidans* (Waksman and Joffe, 1922), one of the few strictly autotrophic bacteria known, was chosen for a study of the metabolism of chemosynthetic cells. The chemosynthetic mechanism and other possible enzymatic reactions in such cells have not been studied extensively. Whereas the general reactions furnishing the chemical energy are known (Starkey, 1925) and data on energy efficiency in growing cultures are available (Waksman and Starkey, 1923), no indications have been obtained thus far as to the nature of the metabolism of the cells.

The requirement of the specific nutrient (in this case sulfur or thiosulfate) for the growth of autotrophic bacteria has led to the conclusion that the metabolism in this form of life is not comparable to that of heterotrophic organisms; that the chemosynthetic mechanism is a unique process furnishing the cell with all necessary materials derived in a different manner than in heterotrophic forms. The inability of this organism to grow on any organic media yet devised lends considerable support to this contention, and one might therefore conclude that the energy released by the oxidation of sulfur (or thiosulfate) is coupled to the synthesis of cell material, but that the energy supply of the organism is directly dependent upon the specific material oxidized. Bömeke (1939) has summarized the pertinent literature concerning the nitrifying bacteria and points out that this viewpoint is the one stressed in most standard textbooks.

It seems probable, on the other hand, that in nature the sulfur-oxidizing bacteria must be able to live for some time in the complete absence of the specific nutrient. And this would lead one to believe that the chemosynthesis supplied the cells with organic storage compounds, the breakdown of which furnishes the energy of maintenance of the organisms and enables them to survive for periods during which the specific nutrient is absent. In this concept the autotrophic bacteria would be considered closely related to the photosynthetic plant, in which a storage of carbonaceous material occurs during illumination and a utilization of that material takes place in darkness.

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Bömeke (1939) has recently been able to show that a definite oxygen uptake may be observed in pure cultures of nitrifying bacteria in the absence of the specific nutrient. This endogenous respiration is exceedingly low and requires long periods of observation.

Apparently critical evidence which would distinguish between the two concepts of autotrophic growth mentioned above would be proof that the endogenous respiration observed is due to the utilization of stored materials and is not dependent upon the incomplete removal of the specific nutrient. It is the purpose of this paper to provide such evidence for *Thiobacillus thiooxidans* and to describe briefly certain modifications in methods of study which are necessary to obtain satisfactory information from this organism.

### Methods

The techniques of growing this organism and the preparation of "resting cell" suspensions have been previously described (Umbreit, *et al.*, 1941). Briefly, they consist of growth of pure cultures on sulfur in a purely mineral medium, filtration of the unused sulfur through cotton, centrifugation of the filtrate in a Sharples centrifuge, and washing of the centrifuged cells in buffer or distilled water. Rather large quantities of media are required to provide sufficient cells for study and under present conditions of culture approximately 5 gm. (wet weight) of cells are obtained from each 100 liters of medium.

Suspensions of *Thiobacillus thiooxidans* prepared in this manner show a measurable oxygen uptake without added sulfur. The  $Q_{O_2}(N)$  (microliters of oxygen per milligram of bacterial nitrogen per hour) of this endogenous respiration is relatively constant with each suspension although it varied from suspension to suspension between the ranges of 10 and 40. The variation among replicates is usually within the accuracy of the instrument (Warburg apparatus) and these cell suspensions can apparently be treated in the same manner as "resting cell" suspensions of heterotrophic bacteria.

Addition of sulfur to such suspensions increases the oxygen uptake from 20 to 100 times even in the absence of  $CO_2$  (KOH in Warburg flask). However, replicates now exhibit a very marked variability; at times apparently identical flasks exhibited variations of over 50 per cent of the average value. Moreover, the  $Q_{O_2}(N)$  after the addition of sulfur does not remain constant but increases markedly over a period of about 4 hours under our usual conditions, at which time it tends to become constant, although occasional intervals may show some variation. This effect is illustrated in columns 1 through 4 of Table I. The oxygen uptake per 30 minute interval rises rapidly which causes a comparable rise in the  $Q_{O_2}(N)$ . The time required for maximum oxygen uptake varies markedly with the concentration of the organism and tends to be much shorter when dilute suspensions are used.

After some study it became apparent that the major cause of these variations was the necessity of direct contact between the sulfur particle and the organism (Vogler and Umbreit, 1941). The inconsistent results observed in the oxidation of sulfur seem to be caused by a variability in the time necessary for the cells to form actual contact with the solid sulfur. This time is easily influenced by a number of accidental

factors; the distribution of sulfur on the surface of the suspension, the amount of sulfur sinking to the bottom, the formation of clumps, etc. Such variability, however, seriously limits the value of the Warburg technique in the study of this oxidation so that studies were directed towards methods of reducing the variability.

Methods which give satisfactory results in the oxidation of sulfur consist of mixing the bacterial suspension, the appropriate buffer, and a suspension of very finely divided sulfur (such as that remaining after the growth of the organism). These are incubated for at least 4 hours and immediately before use the pH is adjusted to the proper value with as little disturbance as possible. An aliquote of this material is placed in the Warburg flask and after equilibration the rates of oxygen uptake are

TABLE I  
*Oxygen Uptake by Thiobacillus thiooxidans on Sulfur*

1	2	3	4	5	6	7	8
Time	Oxygen used	Oxygen per 30 min.	QO <sub>2</sub> (N) over interval	Time	Oxygen used	Oxygen per 15 min.	QO <sub>2</sub> (N) over interval
min.	microliters	microliters		min.	microliters	microliters	
30	11.5	11.5	74	15	18.4	18.4	239
60	47.1	35.6	230	30	36.8	18.4	239
90	94.2	47.1	306	45	55.2	18.4	239
150	240.0	72.9	470	60	73.6	18.4	239
				75	88.9	15.3	199
				90	106.3	17.4	226
				105	124.7	18.4	239
				120	141.1	16.4	213

1.5 cc. suspension 6 (0.21 mg. N/cc.), 1.5 cc.  $\text{M}/10 \text{ KH}_2\text{PO}_4$  (pH 4.8), 100 mg. powdered sulfur on surface (temperature 28°C. KOH in flask).

10 cc. suspension B-3 (0.38 mg. N/cc.), 10 cc.  $\text{M}/10 \text{ KH}_2\text{PO}_4$  (pH 4.0), 10 cc. sulfur suspension (0.207 gm. S/cc.), incubated 20 hrs. 28°C. pH readjusted to 4.0, made to 50 cc. with water, 4 cc. per Warburg flask (temperature 28°C. KOH).

determined. These values should be relatively constant in a series of replicate flasks. Furthermore, since in a given flask the rate of oxygen uptake per unit time is constant, the effect of a variety of materials upon the oxidation could be studied by tipping these materials from the side arm and noting the change in rate of sulfur oxidation.

The protocol of a typical experiment using this type of technique is shown in columns 5 through 8 of Table I in which the relative constancy of oxygen uptake per unit time is evident. The QO<sub>2</sub>(N) on sulfur, while tending to remain relatively constant for each suspension, varies markedly from suspension to suspension. This, of course, is a reflection of the number of living cells in each suspension which tends to vary somewhat. The values for QO<sub>2</sub>(N) given in Table I are not claimed to be maximal values and undoubtedly much larger ones may be obtained after more knowledge of the growth conditions and the respiration of the organism is available. Certain suspensions have been obtained which yield QO<sub>2</sub>(N) values approaching 2000.

*Evidence for Endogenous Respiration*

Proof that endogenous respiration is due to the utilization of organic materials and is not due to traces of sulfur in the suspension is possible from evidence outlined below.

Most suspensions when first prepared are not entirely devoid of sulfur and if they are incubated anaerobically some  $H_2S$  is produced. This is believed by Starkey (1937) to be due to the reaction of glutathione (or similar compounds) in the cells with elemental sulfur. If cultures are allowed to proceed until all of the sulfur is utilized ("starved suspensions"), or if the cells are carefully washed and aerated rapidly for the first few hours after preparation, the production of  $H_2S$  on anaerobic incubation can be eliminated and these suspensions may be regarded as free from sulfur. However, it is impossible

TABLE II  
*Endogenous Respiration of Thiobacillus thiooxidans*

1	2	3	4	5	6	7	8	9	10
Time	Oxygen used	$Q_{O_2}(N)$	$CO_2$ liberated	r.q.	Time	Oxygen used	$Q_{O_2}(N)$	$CO_2$ liberated	r.q.
min.	microliters		microliters		min.	microliters		microliters	
80	13.8	34	13.6	0.98	60	19.6	63	9.3	0.47
210	24.7	22	24.4	0.99	120	36.2	58	19.2	0.57
270	32.8	23	34.4	1.04	190	62.0	63	29.0	0.47
					270	86.1	62	75.0	0.87

1.5 cc. suspension 6 (0.21 mg. N/cc.), 1.5 cc. Same, except trace of sulfur added.  
buffer, pH 4.6, 1.0 cc.  $H_2O$  per flask (temperature 28°C.).

to be sure that every trace of sulfur has been eliminated. Preparations made either by aeration or "starving" show an endogenous respiration in the absence of added sulfur of the usual magnitude ( $Q_{O_2}(N)$  10-40).

If KOH be left out of the Warburg flask, and that respiration compared with replicates containing KOH, there is evidence that  $CO_2$  is produced during the respiration. The r.q. so obtained is close to 1.0. Data for a "starved" suspension are given in columns 1 through 5 of Table II while columns 6 through 10 record the action of the same suspension given a trace of sulfur. In the first case the  $Q_{O_2}(N)$  is within the usual range, and the r.q.'s are about 1.0. When a trace of sulfur is added, the  $Q_{O_2}(N)$  increases beyond the usual value and the r.q. drops to about half its former value; both phenomena are explainable by the oxidation of the small amount of sulfur added. The production of  $CO_2$ , however, regardless of its relative quantity with respect to the oxygen utilized, provides evidence of the utilization of organic materials. Oxygen uptake might indicate sulfur oxidation, but  $CO_2$  could only come from carbon materials.

It is possible to differentiate the endogenous respiration from that on sulfur by means of certain inhibitors of which sodium azide is a striking example. It is evident in Table III that the endogenous respiration cannot be due to traces of sulfur remaining since no inhibition is evident even at very high concentrations of the inhibitor. Proof that the observed endogenous respiration is due to *Thiobacillus thiooxidans* and not to possible heterotrophic contaminants may be obtained from the following considerations. The organism is grown and harvested at a pH of 1.0 to 1.5 which effectively eliminates most contaminants except yeasts and molds. The endogenous respiration observed seems to be independent of the pH and its magnitude is the same at pH 4.8 and at pH 2.0, a character which is most surprising were it due to a contaminant. Finally, glucose may be added to such endogenous suspensions without detectable effect upon the oxygen uptake over a period of at least 8 hours (pH 4.8), so that the respiration observed cannot be due to heterotrophic contaminants but must be due to the autotrophic bacterium. (Glucose

TABLE III  
*The Selective Inhibition of Sulfur Oxidation by Sodium Azide*

Concentration of sodium azide.....	10 <sup>-2</sup> M	10 <sup>-3</sup> M	10 <sup>-4</sup> M
Inhibition of sulfur oxidation, per cent.....	96	90	60
Inhibition of endogenous respiration, per cent....	0	0	0

conc. 0 per cent,  $Q_{O_2}(N)$  43; 0.5 per cent, 40; 1.0 per cent, 38; 2.0 per cent, 44; 4.0 per cent, 37; and 5.0 per cent, 41.) Bömeke (1939), after surmounting very great technical difficulties, reached the same conclusion for *Nitrobacter* and *Nitrosomonas*.

Proof that the respiration observed is yielding energy for the cell can only be obtained indirectly. Suspensions which are to all intents sulfur-free survive at refrigerator temperatures for relatively long periods (several weeks at least). During that time these suspensions have motile forms, they immediately begin to oxidize sulfur when it is supplied to them, and they continually liberate  $CO_2$ . There is, therefore, little doubt that the oxidation of the organic materials within the cell is coupled to the energy needs of the surviving organism.

#### SUMMARY AND CONCLUSIONS

It is shown that there exists in the autotrophic bacterium *Thiobacillus thiooxidans* a measurable oxygen uptake in the absence of the specific nutrient (sulfur). This respiration is shown to be due to the utilization of organic materials which must have been previously synthesized by the chemosynthetic process, providing evidence that autotrophic bacteria contain a dissimilatory

process which involves the breakdown of organic materials and furnishes energy for the maintenance of the cell during periods in which the specific nutrient is absent. This is entirely in accord with the work of Bömeke (1939), who provided similar types of proof for *Nitrosomonas* and *Nitrobacter*. One may conclude, therefore, that autotrophic bacteria possess an endogenous respiration which involves the utilization of previously synthesized organic materials.

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# THE EFFECTS OF HYDROGEN ION CONCENTRATION UPON THE METAMORPHIC PATTERN OF THYROXIN- AND IODINE-TREATED TADPOLES\*

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## I. INTRODUCTION

The effects of external factors upon the metamorphosing action of thyroxin have been but little studied. There have, however, been several papers tending to indicate that certain chemical compounds have an inhibiting effect on the activity of thyroxin while others accelerate it. Thus Zondek and Reiter (1923) reported that tadpoles living in water which contains  $\text{CaCl}_2$  in a concentration of 1 part in 2000 are much less affected by thyroxin in the solution (1 part in 20,000,000) than are tadpoles living in ordinary tap water. On the other hand, they found that  $\text{KCl}$  in concentrations of 1 part in 2000 tends to accelerate the action of thyroxin. These experiments have, however, been repeated by Kosmin and Resnicenko (1927), Hellwig (1936), and Rosen (1938) and no such effects with  $\text{CaCl}_2$  and  $\text{KCl}$  have been obtained.

Abelin (1923) reported that the action of thyroxin is sometimes inhibited by disodium phosphate but found no effects with sodium acid phosphate. He concluded that the  $\text{PO}_4^{--}$  ion is responsible for the inhibition.

This matter was taken up by Rosen (1938) who demonstrated that the important factor in all of the above experiments is the relative acidity of the environmental solution. Inhibition or acceleration of the effects of thyroxin is dependent upon the hydrogen ion concentration in the environmental solution rather than upon the particular ions present. Rosen placed tadpoles in solutions containing the following ions in various combinations:  $\text{Na}^+$ ,  $\text{PO}_4^{--}$ ,  $\text{Cl}^-$ ,  $\text{CO}_3^{--}$ ,  $\text{Ca}^{++}$ ,  $\text{H}^+$ ,  $\text{Mg}^{++}$ ,  $\text{OH}^-$ ,  $\text{C}_6\text{H}_5\text{O}_7^-$ , and  $\text{C}_2\text{H}_3\text{O}_2^-$ . The compounds used were  $\text{Na}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$ ,  $\text{Na}_3\text{PO}_4$ ,  $\text{HCl}$ ,  $\text{CaCO}_3$ ,  $\text{Na}_2\text{CO}_3$ ,  $\text{NaHCO}_3$ ,  $\text{Mg}(\text{OH})_2$ ,  $\text{HC}_6\text{H}_5\text{O}_7$ ,  $\text{HC}_2\text{H}_3\text{O}_2$ . By employing these compounds at various concentrations, a range of hydrogen ion concentrations from pH 4.8 to pH 11.0

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was obtained. It was found that, with the same concentration of thyroxin present in all cases (1:20,000,000), tadpoles showed greatest signs of metamorphosis in the solutions with the lowest pH values and least signs of metamorphosis in the solutions with the highest pH values. At intermediate values the effects were intermediate.

These experiments were carried out on very small groups of tadpoles, only six or seven animals being used for each test, and the experimental period was only 5 days. The results were not expressed quantitatively, no measurements of weight or hind limb length having been made. However, Rosen presents photographs of three animals from each experiment and, since he used a rapidly metamorphosing form (*Rana sylvatica*), the differences are sufficiently well marked to clearly support his general conclusion that "Acidity accelerates and alkalinity retards the metamorphosing action of thyroxin on tadpoles."

No hypothesis has been advanced to account for this observation but it would seem that several possible factors must be considered. Since the thyroxin, when administered in this way must pass through the skin, or other epithelial walls before it can exert its influence, some effect of hydrogen ion concentration upon the permeability may be of importance. On the other hand, it is conceivable that the thyroxin itself is altered by changing hydrogen ion concentration, either by changes in its chemical nature, potency, solubility, or degree of dissociation.

The present experiments have been designed in an attempt to obtain some information concerning these various possibilities. It is clear that, if permeability is a decisive factor, then parallel experiments with the thyroxin injected into the animals rather than administered through the surrounding solution should reveal this fact. Two types of injection experiments are possible. (a) The injection of thyroxin at different hydrogen ion concentrations with the environment at a constant hydrogen ion concentration. (b) The injection of thyroxin at constant hydrogen ion concentrations into tadpoles living in solutions of different hydrogen ion concentrations. The results of these injection experiments can show whether, in Rosen's experiments, hydrogen ion concentration affects the rate of penetration of thyroxin or whether it affects the potency of thyroxin. If there is no effect of hydrogen ion concentration in both of these injection experiments, we can assume that in Rosen's experiment, hydrogen ion concentration affects the rate of penetration of thyroxin. And if there is an effect of hydrogen ion concentration in both of these experiments, then Rosen's experiment can be explained on the basis of change of potency of thyroxin due to change of hydrogen ion concentration.

The problem has been studied quantitatively for both thyroxin and iodine. The use of iodine seemed desirable in view of the great differences in the molecules of thyroxin and iodine. If the effects of hydrogen ion concentration upon

thyroxin are in any way connected with its chemical nature, then they should not be expected to hold in the case of parallel experiments with iodine.

The problems with which the present experiments are concerned can be summarized briefly as follows:

1. Are the potencies of thyroxin and iodine affected by change in hydrogen ion concentration; *i.e.*, does change in hydrogen ion concentration affect the potency of the inducing substances which are injected directly into tadpoles?
2. Are the membrane-penetrating powers of thyroxin and iodine affected by change in hydrogen ion concentration? Does a change in hydrogen ion concentration affect the rate of penetration of an inducing substance which is present in the environmental solutions of tadpoles? Do inducing substances penetrate tadpole membranes more easily at one hydrogen ion concentration than at another and if so, why?
3. Do thyroxin and iodine have similar inducing effects in these experiments?
4. Are solubility, degree of dissociation, size of molecule (properties which are conceivably changed by hydrogen ion concentration changes) involved in the induction of metamorphosis?

## II. Materials and Methods

Tadpoles of *Rana catesbiana* were used in all the experiments. They were collected from the various ponds on the campus of The Johns Hopkins University and the vicinity of Baltimore, but all animals used for a single experiment were taken from a single pond. During the winter months, several hundred animals which had been collected from the same pond during the fall were kept in a large aquarium and used as a storage supply. Before each experiment was begun, the animals were acclimatized in the laboratory for 1 day in tap water. Since the hind limbs are extremely susceptible to inducing effects (Kollman, 1919, and Etkin, 1932, 1935) and can be measured very easily, they are an excellent criterion for the degree of metamorphosis and they have been used as such in these experiments. The animals to be measured were laid on a wet towel and the right hind limb was measured with a pair of calipers to the nearest tenth of a millimeter.

In most of the experiments comparisons were made among five similar groups of animals, each group consisting of nine animals which had hind limbs of varying degrees of development. Because the hind limb response to an inducing stimulus differs according to the initial size, care was taken to select the animals in these groups so that all of the five groups were strictly comparable in length of hind limb.

The animals were distributed so that three were put into each of fifteen finger-bowls. This was done to avoid crowding in the bowls and because, with three animals of different length of hind limbs in a bowl, identification of individual animals was readily made.

From the above description, it is seen that comparisons of response can be made between animals having the same length of hind limbs, but receiving different treatments. Since there are individual variations among animals, conclusions are drawn

from the comparison of the average response of nine animals receiving a certain type of treatment with the average response of a similar group of nine animals receiving a different type of treatment.

In some experiments, the inducing substance, thyroxin or iodine, was contained in the environmental solution while in others it was injected directly into the animals. The hydrogen ion concentration of environmental solutions and injected material was varied independently.

For injecting into the tadpoles the inducing substances used in the experiments, a hypodermic needle was introduced into the right body wall, at the point where the tail joins the body and about 1 millimeter dorsal to the hind limb. The needle was pushed anteriorly into the coelom, where the fluid was emptied.

Fresh stock solutions of thyroxin or iodine were made up for each experiment. The thyroxin was made up by dissolving 10 mg. of thyroxin in 2 cc. of 0.1N KOH and diluting with 98 cc. of distilled water. This produced a solution containing 1 part of thyroxin in  $10^4$  parts of water. The iodine stock solution contained 0.254 gm. of iodine in 40 cc. of 86 per cent alcohol, making a 0.025 M solution of iodine. In the experiments, these stock solutions were diluted to the desired concentrations with tap water. (See individual experiments.)

The solutions in which the animals were confined, designated as environmental solutions, were changed daily. This was done both in experiments in which the environmental solutions consisted of tap water and those in which it consisted of buffer solutions. In experiments calling for environmental solutions at different hydrogen ion concentrations, very dilute McIlvane (disodium phosphate-citric acid) buffers were used between pH 4 and 7.5 and Kolthoff and Vlesschouwer (soda-borax) buffers were used at pH 8.5.

These buffer solutions were made up in lots of 2400 cc. Thyroxin or iodine was added to the buffer solutions, giving a solution whose hydrogen ion concentration and thyroxin or iodine content were known. The 2400 cc. of buffer solution containing thyroxin or iodine were distributed into each of three finger-bowls so that there were 800 cc. of solution in each. Hydrogen ion concentrations were ascertained by means of the type K modification of the Leeds and Northrup potentiometer, using the quinhydrone electrode.

The first group of experiments, essentially six types, were performed as follows:

A. Experiments in which animals were injected with 3/20 cc. of thyroxin solution (1 part in  $10^4$ ) at various hydrogen ion concentrations, the environmental solutions being unbuffered tap water (average pH 6.9).

B. Experiments in which animals were kept in solutions buffered at different hydrogen ion concentrations and injected with 1/5 cc. of stock solution thyroxin (pH 8.7).

C. Experiments in which animals were kept in environmental solutions buffered at different hydrogen ion concentrations and containing 1 part of thyroxin in  $8 \times 10^6$  of solution.

D. Experiments in which animals were injected with 5/32 cc. of 0.0008 M iodine solutions at various hydrogen ion concentrations, the environmental solutions being unbuffered tap water (average pH 6.9).

E. Experiments in which animals were kept in solutions buffered at different hydrogen ion concentrations and injected with 5/32 cc. of 0.0008 M iodine.

F. Experiments in which animals were kept in environmental solutions buffered at different hydrogen ion concentrations and containing 0.000 006 25 M iodine.

It will be seen that these six types of experiments provided, for both iodine and thyroxin, three general modes of treatment: (1) environmental solutions at constant hydrogen ion concentration, inducing agent injected at different hydrogen ion concentrations;<sup>1</sup> (2) environmental solutions at different hydrogen ion concentrations and containing the inducing agent in solution; (3) environmental solutions at different hydrogen ion concentrations, inducing agent injected at constant hydrogen ion concentration.

A second group of experiments was performed to further investigate and clarify these findings. Individual variations in the methods employed accompany the experiment in question.

No untreated controls were included in the experiments, since the only purpose was to compare the effects of various hydrogen ion concentrations on induced development and since natural growth over such short periods as were involved in the experiments is too small for measurement.

### III. EXPERIMENTAL

In early experiments it became apparent that the increase in length of hind limb shown by a tadpole under a given treatment, varied considerably depending upon the initial length of the hind limbs. Thus tadpoles with an initial difference of 1 or 2 mm. in the length of the hind limbs show strikingly different responses to the same treatment. Since the validity of the experiments is dependent upon measurements of hind limb growth as a criterion of the effectiveness of the treatments used, preliminary experiments were performed to ascertain just how far such initial differences might affect the results.

The results of these experiments, the data of which are not given here, show clearly that a very close relationship does exist between the response of the hind limbs and their initial size. (The coefficient of correlation was found to be 0.91 in one experiment and 0.84 in another.) It was therefore necessary, in all later experiments to select very carefully animals with exactly the same

<sup>1</sup>In this connection, no claim is made that a substance injected at a particular hydrogen ion concentration remains at that hydrogen ion concentration after being injected into the animal. As will be seen from the experimental results, a substance sometimes does differ in its effects when injected at initially different hydrogen ion concentrations.

initial length of hind limb in order to insure strictly comparable results in all experiments.

### *A. The Effects of Injection of Thyroxin at Diverse Hydrogen Ion Concentrations*

#### *(a) Procedure*

On Jan. 9, 1940, a set of experimental animals having hind limbs ranging from 3.0 to 6.0 mm. was injected with thyroxin buffered at various diverse hydrogen ion concentrations. Five groups of nine animals were used as explained under Materials and methods. The members of the various groups were injected with thyroxin solutions buffered at pH 4.5, 5.3, 6.7, 8.0, and 9.0.

On Jan. 16, when one animal died, the experiment was terminated and the limbs of all animals were measured. The results are given below.

TABLE I

*The Increase after 7 Days in the Length of Hind Limbs of Tadpoles Injected with Thyroxin at Different Hydrogen Ion Concentrations*

Initial length	Increase in length				
	[H <sup>+</sup> ] in pH units of thyroxin solutions injected				
	4.5	5.3	6.7	8.0	9.0
mm.	mm.	mm.	mm.	mm.	mm.
6.0	5.8	5.7	5.9	5.6	5.8
5.0	4.8	4.8	4.6	5.2	5.2
3.8	4.8	4.3	4.6	4.4	4.9
3.2	4.1	4.2	3.9	3.9	4.2
3.2	4.1	3.9	3.0	3.5	4.0
3.1	3.8	3.7	4.1	3.6	3.9
3.0	3.8	3.3	3.8	3.7	3.7
3.0	5.0	3.2	3.0	3.3	3.0
3.0	3.0	3.0	3.2	3.2	3.0
Average.....	4.2	4.0	4.0	4.0	4.2

#### *(b) Results*

Table I shows that there is no significant difference in the response shown by the animals which received these different treatments. Thus the response of the tadpoles to injected thyroxin is not dependent upon the hydrogen ion concentration of the thyroxin solution injected, and it appears that the potency of the inducing substance in this experiment has not been affected.

### *B. The Effects of Varying the Hydrogen Ion Concentration of the Environment and Injecting Thyroxin at Constant Hydrogen Ion Concentration*

#### *(a) Procedure*

In these experiments the hydrogen ion concentration of the environment was varied and that of the thyroxin injected into the animals was the same for all animals.

All animals were injected with 3/20 cc. of 1 part in  $10^4$  thyroxin (pH 8.7) and put into environmental solutions with varying hydrogen ion concentrations. One experiment was started on Dec. 5, 1939, and was concluded on Dec. 11. The initial length of the hind limbs in this experiment varied from 3.4 to 5.9 mm.

A second experiment performed May 7 to 11, was carried out on younger animals with limbs ranging from 2.1 to 3.2 mm. in length.

TABLE II

*The Increase after 6 Days in Length of Hind Limbs of Tadpoles Living in Solutions of Different Hydrogen Concentrations and Injected with Thyroxin*

Initial length	Increase in length				
	[H <sup>+</sup> ] of environmental solutions in pH units				
	4.4	5.3	6.2	7.4	8.5
mm.	mm.	mm.	mm.	mm.	mm.
5.9	3.0	5.5	5.7	5.3	5.4
5.2	5.5	5.8	3.7	5.5	5.5
4.9	4.4	4.8	4.2	4.3	4.3
4.6	5.4	5.1	5.3	5.0	4.9
4.2	4.7	4.2	4.5	4.6	4.2
4.0	4.7	3.4	4.0	4.1	3.4
3.8	3.8	4.2	3.7	4.1	4.0
3.7	4.1	4.4	5.4	5.5	3.2
3.4	3.4	3.5	3.8	3.5	3.5
Average . . .	4.6	4.5	4.7	4.7	4.5

(b) Results

The results of Experiment 1 given in Table II show that the average increase in the variously treated animals is fairly constant. Experiment 2 gave similar results. Thus it appears that the hydrogen ion concentration of the environment has no effect upon the response of tadpoles to equal quantities of injected thyroxin.

*C. The Effect of Environmental Solutions Having Diverse Hydrogen Ion Concentrations upon the Inducing Action of Thyroxin in Solution*

(a) Procedure

This experiment was performed April 27 to May 4, 1939. The length of the hind limbs ranged from 1.7 to 3.3 mm. In this experiment the members of each group did not correspond exactly in length of hind limb at the start of the experiment. The averages for each group, however, were the same. The animals were kept for 7 days in solutions containing thyroxin (1 part in  $8 \times 10^6$  solution) buffered at diverse hydrogen ion concentrations.

TABLE III

*The Increase after 7 Days in the Length of Hind Limb of Tadpoles Living in Thyroxin Solutions at Different Hydrogen Ion Concentrations*

		Limb length										Average increase
		mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
[H <sup>+</sup> ] of environmental solution in pH units	4.2	Initial	3.3	2.9	2.7	2.7	2.5	2.4	2.1	2.0	1.7	3.6
		Increase	6.0	3.1	3.7	3.5	3.6	3.3	2.6	2.4	2.2	
	5.2	Initial	3.2	2.9	2.7	2.6	2.5	2.4	2.1	1.9	1.7	3.2
		Increase	3.0	3.3	4.1	3.0	2.2	3.0	1.8	2.2	2.0	
	6.3	Initial	3.1	2.8	2.7	2.7	2.4	2.4	2.2	2.0	1.7	3.1
		Increase	3.8	3.9	3.3	3.1	3.6	3.0	3.0	2.4	1.8	
	7.4	Initial	3.2	2.9	2.7	2.7	2.6	2.4	2.2	1.8	1.7	2.9
		Increase	3.9	4.0	3.7	3.8	3.6	2.9	2.3	1.5	1.6	
	8.8	Initial	3.3	2.9	2.7	2.7	2.4	2.3	2.2	1.9	1.7	2.4
		Increase	3.7	3.3	3.0	2.1	2.3	2.2	1.9	1.4	1.3	

(b) Results

It can be seen in Table III that the greatest average increase in hind limb length occurs in the animals living in the more acid solutions. Since the response to thyroxin is proportional to the amount which the animal receives and since it is independent of the hydrogen ion concentration of the thyroxin solution of the environmental solutions (experiments of Sections A and B), then in these experiments the effect of hydrogen ion concentration must be in regulating the amount of thyroxin which enters the animal body.

Large amounts of water pass in and out of the tadpole body through the integument and animal membranes. This experiment indicates that the hydrogen ion concentration of the solution in which the tadpole is living in some way affects the rate of passage of thyroxin through the integument and animal membranes.

Thus the greatest passage of thyroxin into the animal occurs at the higher hydrogen ion concentrations (lower pH values).

*D. The Effects of Injection of Iodine at Different Hydrogen Ion Concentrations*

(a) Procedure

These experiments are like those outlined in Section A, but iodine is used instead of thyroxin. On Feb. 6, 1940, the experimental animals were injected with iodine at various hydrogen ion concentrations and put into finger-bowls containing tap water. In this experiment, each group contained twelve animals instead of the customary nine. The hind limb lengths ranged between 2.8 and 3.0 mm. In this series there were 30 animals which had an initial hind limb length of 2.8 mm. but differed greatly in body size. These were equitably distributed into five finger-bowls according to body size. In this experiment, then, one series of animals con-

sisted of five finger-bowls containing six animals having the same length of hind limb but different body sizes.

The animals were injected on Feb. 6, Feb. 17, and Feb. 24 with 5/32 cc. of 0.0008 M iodine at a hydrogen ion concentration of pH 4.5, 5.6, 6.6, 7.5, and 8.6. The experiment ended on Feb. 29.

TABLE IV

*The Increase after 23 Days in the Length of Hind Limb of Tadpoles Injected with Iodine at Different Hydrogen Ion Concentrations*

Initial length	Increase in length				
	[H <sup>+</sup> ] of injected iodine solutions in pH units				
	4.5	5.6	6.6	7.5	8.6
mm.	mm.	mm.	mm.	mm.	mm.
2.8	3.4	6.0	6.4	1.6	1.0
2.9	0.8	5.3	3.7	1.5	0.6
2.8	0.7	3.2	3.4	1.0	0.1
2.8	0.6	3.2	3.1	0.9	0.1
2.8	0.2	1.6	3.0	0.9	0.1
2.8	0.2	0.4	2.1	0.4	0.0
2.9	0.1	2.9	4.5	0.9	0.8
2.9	0.0	1.9	3.3	0.3	0.4
2.9	0.0	1.8	2.7	0.1	0.1
3.0	1.0	1.9	1.2	0.6	0.7
3.0	0.1	1.8	1.1	0.0	0.3
3.1	3.2	4.3	4.9	3.7	2.1
Average	0.9	2.9	3.3	0.9	0.5

(b) *Results*

The results, indicated in Table IV show that the inducing potency of injected iodine, unlike that of thyroxin, is dependent on its hydrogen ion concentration. Animals injected with iodine at pH 4.5, 7.5, and 8.6 showed hardly any response but those injected with iodine at pH 5.6 and 6.6 did respond significantly.

*E. The Effects of Environmental Solutions of Diverse Hydrogen Ion Concentrations upon the Influence of Injected Iodine*

(a) *Procedure*

On Nov. 14, 1939, animals having hind limbs of 4.8 to 8.0 mm. were injected with 5/32 cc. of 0.0008 M iodine and put into environmental solutions at diverse hydrogen ion concentrations. The animals were injected again on Nov. 21 and were measured on Nov. 25 and Nov. 30.

A second experiment was performed Oct. 24 to Nov. 4 with animals ranging in length of hind limb from 3.2 to 5.4 mm. The animals were injected with 1/5 cc. of 0.0005 M iodine on Oct. 24 and 1/5 cc. of 0.0012 M iodine on Oct. 29.



TABLE V

*The Increase after 9 and after 12 Days in the Length of Hind Limbs of Tadpoles Living in Solutions of Different Hydrogen Ion Concentrations and Injected with Iodine*

Initial length	Increase in length									
	[H <sup>+</sup> ] of environmental solutions in pH units									
	4.2		5.5		6.2		7.5		8.5	
	A	B	A	B	A	B	A	B	A	B
mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
8.0	4.1	6.1	5.6	7.6	5.9	7.3	5.1	7.4	3.1	5.9
7.3	3.7	4.9	3.6	5.4	4.1	6.1	1.9	3.4	5.6	7.2
7.0	3.6	5.1	2.4	5.0	3.8	5.7	2.3	3.9	2.4	4.3
6.8	3.8	4.6	4.1	5.6	5.3	7.4	4.3	5.9	4.1	6.6
6.0	3.2	4.9	3.9	6.1	4.9	6.0	2.9	4.3	4.3	7.7
5.7	3.0	5.3	3.1	5.2	3.2	5.7	2.5	4.9	0.7	2.5
4.9	2.6	4.1	3.2	4.8	6.0	8.5	3.3	5.3	2.1	3.7
4.8	2.4	3.1	2.3	4.1	3.2	5.2	3.3	4.6	1.2	3.0
4.8	3.4	5.5	3.2	4.3	2.7	4.2	3.2	5.2	1.4	3.0
A, average....	3.3		3.5		4.3		3.2		2.6	
B, average....		4.8		5.3		6.2		5.0		4.9

A, measurements for Nov. 25.

B, measurements for Nov. 30.

### (b) Results

Table V, containing the measurements for Experiment 1 made on Nov. 25 and Nov. 30 shows that the hydrogen ion concentration of the environmental solution does affect the inducing action of injected iodine. On plotting the average increase in length of hind limbs against pH values of the environmental solutions, the resultant curve resembles that of the preceding experiment. In both cases the curve is low at pH 4.5 and 8.5 and reaches a maximum between pH 6.2 and 6.6. However, the slope of the curve is much greater in the preceding experiment, the results being more marked when the iodine is injected directly. Although the hind limbs have increased in size between Nov. 25 and Nov. 30, the curves for these two dates are very similar.

The results of Experiment 2 agree with those of 1 in spite of the fact that little growth of the hind limbs was obtained due to the short duration of the experiment.

It can be concluded from these experiments that the potency of iodine is not only dependent on the hydrogen ion concentration of the iodine when injected but also on the hydrogen ion concentration of the solutions in the immediate environment of the animals.

Presumably, this is due to the fact that the environmental solutions are continually being taken into the animal. Since the potency of iodine is dependent upon its hydrogen ion concentration, the environmental solutions, being continually carried into the animal can conceivably act on the potency of the iodine. Thus environmental solutions of different hydrogen ion concentrations have diverse effects.

*F. The Effects of Environmental Solutions at Diverse Hydrogen Ion Concentrations, Containing Iodine*

*(a) Procedure*

On Oct. 2, 1939, the experimental animals ranging from 3.0 to 5.9 mm. in length of hind limbs were put into solutions of different hydrogen ion concentrations containing 0.000, 006, 25 M iodine. The animals were measured on Oct. 21.

TABLE VI

*The Increase after 19 Days in the Length of Hind Limbs of Tadpoles living in Solutions of Different Hydrogen Ion Concentrations Containing Iodine*

Initial length	Increase in length				
	[H <sup>+</sup> ] of environmental solutions in pH units				
	4.5	5.3	6.2	7.5	8.5
mm.	mm.	mm.	mm.	mm.	mm.
5.9	0.0	0.7	2.4	4.0	1.8
5.2	0.0	0.6	3.0	5.9	3.7
4.8	0.0	0.5	4.0	4.0	1.0
4.1	0.0	0.4	3.0	4.0	1.2
4.0	0.0	0.7	2.8	3.7	0.8
3.9	0.0	0.3	3.2	2.1	1.3
3.2	0.0	0.6	3.0	3.5	1.7
3.1	0.0	0.6	1.9	2.8	0.7
3.0	0.0	0.3	2.1	2.5	0.8
Average . . . .	0.0	0.5	2.8	3.6	1.4

Although the experiment took 19 days, the animals at pH 4.5 all died after 10 days.

*(b) Results*

After 10 days all the animals at pH 4.5 died without any growth of hind limbs. The animals at pH 7.5 showed a measurable increase in length of hind limbs, but those at pH 6.8 and 5.2 grew very little. The results of this experiment are given in Table VI. Plotting average increase in length of hind limbs against hydrogen ion concentration of the environmental solutions shows a curve with a deep slope at the hydrogen ion concentrations above and below pH 7.5.

The previous experiments with iodine (Sections D and E) have shown that the potency of injected iodine is affected by the hydrogen ion concentration of the injected iodine and the hydrogen ion concentration of the environmental solution. Graphs for these two experiments show the same maximum points of response although in one type of experiment the hydrogen ion concentration change is in the environment and in the other type in the substance injected into the animal. These results differ, however, from the results of the experiments in this section (Section F) in the maximum point of response. The essential experimental difference between the experiments of Section F and those of Sections D and E is the penetration of the integument by the inducing substance in the experiments of Section F. Thus the difference between the results of this experiment and the previous iodine experiments may in some way be due to the two factors which are at work here, namely potency and the penetration of the integument as contrasted with one factor involved in the previous experiments, namely potency.

From the iodine experiments it can be concluded that iodine solutions, unlike thyroxin solutions, vary in their power of metamorphic induction according to the hydrogen ion concentration.

*G. Investigations into the Nature and Cause of the Effects of Hydrogen Ion Concentration on Thyroxin-Induced Metamorphosis*

It has been shown that the development of tadpoles in environmental solutions containing thyroxin is dependent upon the hydrogen ion concentration of that environment. Further experimentation indicated that this effect is not due to a difference in the chemical nature of the thyroxin, but to a difference in the rate of penetration of thyroxin into the animals under these diverse conditions. It is now desirable to attempt to ascertain the factors involved in this difference in penetration rate under differing conditions of acidity and alkalinity.

One of the most logical suspicions is that the metabolic activity of the animal is dependent upon the hydrogen ion concentration of the environment. A change in metabolic activity can bring about a change in the rate of intake of environmental fluid and its dissolved substances and such a change could conceivably account for the differential growth of tadpoles in solutions of thyroxin at different hydrogen ion concentrations. The following experiments were undertaken in an attempt to test this hypothesis.

*(a) Procedure*

Respiration-metabolism tests were made upon the same twelve tadpoles at different hydrogen ion concentrations. The animals ranged in weight from 1.9 to 4.1 gm. They were kept in tap water for 1 week before being tested at another hydrogen ion concentration. The tests were made in unbuffered tap water at pH 6.9 and in buffered tap water at pH 4.3 and 8.4.

The apparatus used in this experiment is diagrammed in Fig. 1. Water (*w*) at a known hydrogen ion concentration was put into a 20 liter carboy and oxygen was allowed to bubble through slowly. A manometer (*m*) was connected to the carboy so that the oxygen pressure in the carboy was maintained at 795 mm. of mercury. The water was allowed to siphon down into each of four chambers (*a*); three of these contained experimental animals while the fourth served as a control. These chambers were mounted in an aquarium with adequate facilities (*s, h, t, r*) for maintaining a constant temperature of 24.5°C. The chambers were connected with two parallel tubes (*ct, ot*) inclined at an angle of 45°. One tube (*ot*) was an overflow tube, the other (*ct*) a collecting tube. A steady flow of water was maintained through the

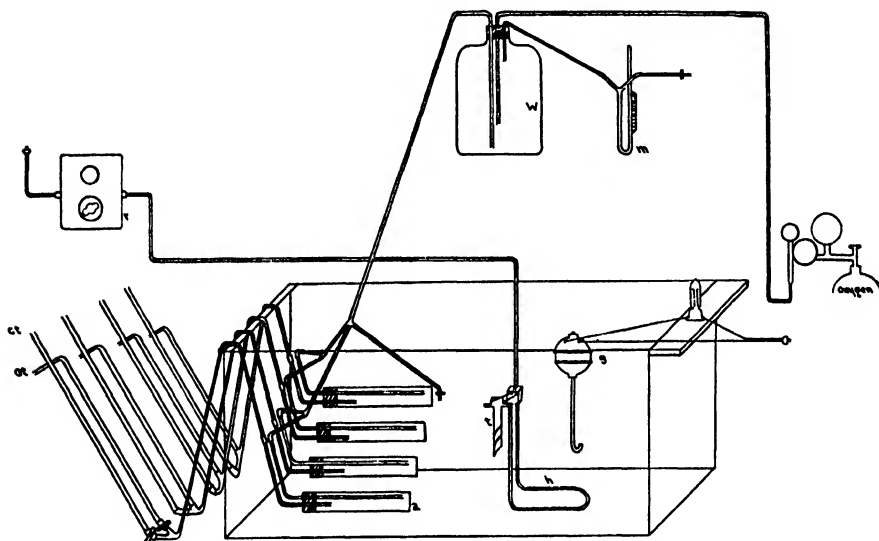


FIG. 1. Apparatus for respiratory metabolism test. *a*, animal chamber; *h*, heater; *m*, manometer; *r*, relay; *s*, stirrer; *t*, thermoregulator; *w*, water source; *ct*, collecting tube; *ot*, outflow tube.

overflow tube for 4 hours at 100 cc. per hour during which time the tadpoles were acclimatized and the entire system reached an equilibrium. Then the overflow tube was closed and water was allowed to flow up the collecting tube at the same rate of flow. This water was collected under oil and analyzed for oxygen content. The oxygen content of the control minus the oxygen content of the water which had flowed past the tadpoles was considered to be the amount of oxygen consumed by the tadpole.

Measurements of dissolved oxygen in water were made by the Thresh iodometric method of titration. This method has been described by Sutton (1896). It was found to be accurate and yet more convenient than the Winkler method generally used by biologists. It is based on the fact that KI when mixed with sulfuric acid and sodium nitrite liberates iodine in proportion to the amount of dissolved oxygen present. The liberated iodine can then be calculated by titration with sodium thiosulfate.

*(b) Results*

Over one hundred and forty respiration-metabolism tests were performed, the results of which are not shown here in table form. At pH 8.3 the rate of oxygen consumption in mg./hr./gm. was  $0.175 \pm 0.0136$  (standard error), at pH 6.9 it was  $0.317 \pm 0.0445$ , and at pH 4.3 it was  $0.441 \pm 0.0195$ . These results show clearly that the hydrogen ion concentration of the surrounding medium has a profound effect on the rate of respiratory metabolism of the tadpole. The greatest extent to which these figures can be wrong (three times the standard error) still shows no overlapping of numbers at pH 4.3 and pH 8.3. It would seem that these findings may provide the explanation for the effect of hydrogen ion concentration on thyroxin-induced metamorphosis. It is therefore postulated that increase in the metabolic rate results in increase in the corresponding intake of the environmental water with the result that a greater amount of dissolved thyroxin penetrates the integument in a given time. The reverse is true when the metabolic rate is decreased. Keeping tadpoles in thyroxin solutions at different hydrogen ion concentrations therefore results in differential growth.

*H. The Effects of Hydrogen Ion Concentration on Normal Metamorphosis*

The effect of hydrogen ion concentration on the rate of metabolic activity in thyroxin-treated animals immediately gives rise to the suspicion that normal metamorphosis may also be affected by this factor. It would be expected that animals engaged in active secretion of thyroid hormone would secrete in accordance with their rate of metabolism, and also that animals with depleted stores of hormone would replenish their supply in accordance with their metabolic rate. In order to test the validity of these ideas, large tadpoles, nearing the metamorphic crisis were used. It is obvious that small tadpoles cannot be utilized because their normal growth is so slow as to make their use impractical in a study of this kind. On the other hand a difficulty is encountered in obtaining large numbers of old tadpoles in exactly the same stage of hind limb development. This difficulty becomes of little importance, however, in view of the fact that animals nearing the metamorphic climax have reached a point of maximum responsiveness. That is, response of the hind limbs to growth-inducing substances is no longer significantly dependent on their initial size. Therefore a comparison of the growth of hind limbs among animals differing by not more than 1 mm. in initial hind limb length is wholly justifiable.

The following experiments were performed at different seasons of the year. The first experiment was performed on winter tadpoles with depleted thyroids. These animals probably had to synthesize their hormone from iodine taken in in small amounts from the environment. The second experiment was performed on summer tadpoles actively engaged in secreting the thyroid hormone.

*Experiment 1**(a) Procedure*

Forty tadpoles were selected that had hind limbs which varied between 15 and 37 mm. in length. These were divided into five groups of eight, the members of each group not varying in hind limb length from the members of any other group by more than 1 mm. The animals were kept at different hydrogen ion concentrations for 28 days (Jan. 10 to Feb. 7). Their only source of food was *Elodea*, *Spirogyra*, and bread.

TABLE VII

*The Effect of Hydrogen Ion Concentration on the Rate of Growth of the Hind Limbs of Tadpoles*

			Limb length										Average increase
			mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
[H <sup>+</sup> ] of environmental solution in pH units	4.3	Initial	31.8	23.3	21.0	16.1	36.1	26.1	22.5	15.0			
		Increase	2.3	6.4	2.0	0.6	1.4	2.2	1.0	0.5			2.1
	5.3	Initial	32.0	24.0	20.8	16.1	36.8	26.6	23.2	15.0			
		Increase	0.6	0.8	1.3	1.0	1.4	2.4	1.2	0.0			1.1
	6.2	Initial	32.1	24.2	20.7	16.1	35.2	26.0	22.9	15.0			
		Increase	0.8	1.8	0.0	0.2	2.6	1.3	0.9	0.7			1.1
	7.3	Initial	31.7	23.7	20.6	16.1	35.8	26.0	22.2	15.0			
		Increase	0.1	0.6	0.3	0.1	2.2	0.1	0.7	0.7			0.6
	8.6	Initial	31.8	23.6	20.1	16.1	36.2	26.1	23.0	15.0			
		Increase	0.0	1.2	0.0	0.9	0.1	0.7	0.3	0.1			0.4

*(b) Results*

Development was extremely slow due to the fact that the thyroid gland was depleted of hormone following the active growing period of the summer months. This, however, does not detract from the significance of the results which show five times as much growth at pH 4.3 as at pH 8.6. These results are shown in Table VII. Differential growth seemingly results from the control which metabolic activity exerts on the amount of iodine which enters the animal from the environment. This explanation is based on a supposition that the iodine of the environment (derived probably from the *Spirogyra* and *Elodea*) exists in a form whose inducing effect is not dependent on hydrogen ion concentration. On the other hand it is not impossible that animals nearing the metamorphic climax can draw on other organs of the body for a supply of iodine. Either explanation requires the interpretation of differential growth in this experiment as due to an effect of the differential metabolic rates found in these animals.

*Experiment 2**(a) Procedure*

Forty-five tadpoles were selected which had hind limbs varying from 4.3 mm. to 14.1 mm. in length. Five groups of nine animals were made, the members of each group differing but slightly from those of another group inasmuch as these animals were not all near the metamorphic climax. The various groups were kept at different hydrogen ion concentrations for 25 days (June 19 to July 14).

TABLE VIII

*The Effect of Hydrogen Ion Concentration on the Rate of Growth of the Hind Limbs of Tadpoles*

		Limb length										Average increase
		mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
[H <sup>+</sup> ] of environmental solution in pH units	4.3	Initial	14.1	9.3	8.3	8.0	7.0	6.0	5.0	4.9	4.3	8.1
		Increase	20.1	17.7	13.4	7.9	2.6	7.8	0.4	0.0	2.8	
	5.3	Initial	14.1	9.3	8.3	7.9	7.0	6.1	5.0	4.9	4.3	8.1
		Increase	22.7	22.5	2.6	1.9	18.9	1.3	0.1	0.4	2.5	
	6.2	Initial	14.2	9.3	8.3	7.9	7.0	6.0	5.0	4.9	4.3	8.1
		Increase	20.9	16.4	27.5	0.9	3.9	0.4	2.2	0.4	0.0	
	7.3	Initial	14.1	9.3	8.3	7.9	7.0	6.1	5.0	4.9	4.3	3.6
		Increase	19.6	5.5	2.8	0.0	1.4	1.3	1.0	0.0	0.4	
	8.6	Initial	14.1	9.3	8.3	8.0	7.0	6.0	5.0	4.9	4.3	3.1
		Increase	16.8	1.1	3.9	2.7	1.2	1.2	0.0	0.0	0.4	

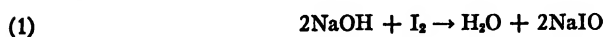
*(b) Results*

These animals present a wide degree of variation in the responses of individual tadpoles. Probably this is due to the fact that some animals have acquired and stored a greater amount of hormone than have others during the winter and spring. The results of this experiment, shown in Table VIII, point to greater response among the larger limbed tadpoles. Being older and larger, they would be expected to have stored larger amounts of iodine than the smaller limbed tadpoles. Generally speaking, the variability in response of the tadpoles depends on the intensity and rigidity of environmental stimuli. The response of the tadpole to iodine is less variable in a buffered solution than in an unbuffered one because of the relative rigidity of the buffered environment. Then also, the more potent the inducing stimulus, the less variable the response. The response of tadpoles to thyroxine is less variable than the response to iodine, and this response is in turn less variable than the response to the normal growth stimuli. The effects of hydrogen ion concentration in this experiment may be interpreted as resulting from a metabolic control of the release of the thyroid hormone. The average growth of the nine animals at pH 4.3 is over twice as great as that at pH 8.6.

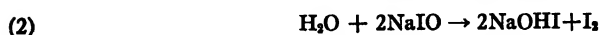
*I. Investigations into the Nature and Cause of the Effects of Hydrogen Ion Concentration on Iodine-Induced Metamorphosis*

Since we have now found a possible explanation for the effects of hydrogen ion concentration on thyroxin-induced metamorphosis, it is well to turn to an investigation of iodine-induced metamorphosis. This we know to be different from thyroxin-induced metamorphosis in view of the experimental results already presented. It will be remembered that the effects of hydrogen ion concentration upon iodine-induced metamorphosis are such that injected iodine at pH 4.3 or at pH 8.5 causes little or no development of the hind limbs while at pH 6.7 there is much development. These results differ markedly from those obtained when thyroxin is used as the inducing agent and it is therefore clear that any explanation for the effects of hydrogen ion concentration on thyroxin-induced development would be unsatisfactory for iodine-induced development. In fact it seems clear that effects of hydrogen ion concentration upon iodine-induced metamorphosis are concerned with changes in the chemical state of the iodine itself rather than changes in the amount of the inducing agent which enters the tadpole body. In the following paragraphs are listed a few equations which illustrate the kind of chemical changes which occur when iodine is put into water and buffered at different hydrogen ion concentrations (Ephraim, 1934).

In strongly alkaline solutions, hypiodite is formed. The reaction is complete, and accounts for the fact that the starch test for iodine cannot be used in strongly alkaline solution.



This reaction is reversible and in a weakly alkaline solution the formation of hypiodite is not complete, so that the starch test for iodine can be used.



Due to the removal of  $\text{H}^+$  ions in alkaline solutions, iodine reacts with water to form salts of the oxy-acids.



The reverse reaction takes place in acid solutions.

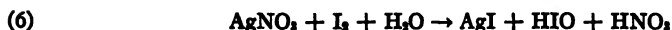


The hydrolysis of iodine is favored by alkaline solution. The equilibrium point in this reaction lies well to the left and only small amounts of these substances are produced.



The yield of IOH can be increased by adding either silver salts or mercuric oxide.





Hypiodous acid is extremely unstable forming HI and HIO<sub>3</sub> readily.



This reaction is very slow in dilute solution.

From the above reactions, it is evident that when iodine is put into water, it assumes a variety of chemical forms, the predominance of any one form being dependent on the hydrogen ion concentration. It is logical then to suspect that differential growth of tadpoles in iodine at different hydrogen ion concentrations is due to the presence of a variety of chemical forms of iodine and that these forms are not all equal in their ability to induce development. The following experiments with iodine were designed to investigate such a possibility.

#### *Experiment 1*

##### *(a) Procedure*

Three groups of animals were selected whose hind limbs varied between 3.2 mm. and 4.5 mm. in length. Each group was put into a solution buffered at pH 6.8 and containing iodine at a concentration of  $6.25 \times 10^{-6}$  M. The iodine was supplied in three chemical forms, as elemental iodine, as potassium iodide, and as potassium iodate. In this way the effect of iodine, iodide, and iodate can be compared with reference to their ability to induce development.

##### *(b) Results*

The results of this experiment show clearly that the form in which inorganic iodine is administered determines the degree of growth response in the tadpole. Elemental iodine buffered to pH 6.8 has been shown to be very efficient (Experiment F). In this experiment it can be seen that it is much more efficient than either iodide or iodate. Iodate in the same concentration shows no ability whatsoever to induce development while iodide shows only a very slight ability. The average growth of the hind limbs of nine animals in iodine solution was 2.0 mm., those in iodide solution showed an increase of only 0.1 mm. during the same period, and those in iodate solution failed to grow at all.

#### *Experiment 2*

##### *(a) Procedure*

Two groups of tadpoles were selected which had hind limbs varying between 2.9 mm. and 6.0 mm. in length. One group of animals was put into a solution buffered at pH 6.8 and containing  $6.25 \times 10^{-6}$  M iodine. The other group of animals was put into a similar solution containing in addition  $2 \times 10^{-7}$  M AgNO<sub>3</sub>. The silver nitrate was added so as to remove iodide ions and increase the production of iodite at the expense of iodine (equation 6). Since we know that elemental iodine buffered at pH 6.8 is much more potent than iodide, the reduction in elemental iodine with the production of iodite should indicate the relative potency of the elemental iodine as compared with iodite.

*(b) Results*

In this experiment the amount of iodite produced in the solution containing silver nitrate was rather small. This was necessary because of the fact that silver nitrate is a very toxic substance and can be added in only small quantities if the tadpoles are to survive. This means that equation 6 could by no means be brought to completion. The experimental results show that an iodine solution at pH 6.8 is far more potent than an iodite solution at the same hydrogen ion concentration. The results do not of course indicate to what extent iodite does induce development, if at all since the iodite-containing solution contained even more iodine than iodite. The average growth in the length of the hind limbs of tadpoles was 2.3 mm. in the elemental iodine solution and 1.7 mm. in the solution containing iodine and iodite.

*Experiment 3*

When iodine is added to water a variety of ions is formed and the concentration of the individual ions is dependent upon the hydrogen ion concentration of the solution. It can be seen from equation (4) that an increase in hydrogen ion concentration hinders the formation of iodide and iodate, and from equation (5) that an increase in hydrogen ion concentration hinders the formation of iodite. Thus increased acidity results in an increased concentration of free iodine. We have already seen that iodine solutions are less and less potent as inducing agents as the hydrogen ion concentration is increased from the neutral point and one must assume that this is due to the fact that free iodine of itself is not a potent inducing substance. This would seem to indicate that some substance which is produced at the neutral point must be present along with free iodine in order for the iodine to have its full effect. Among the substances produced at the neutral point are iodide, iodate, and iodite. We know that iodide reacts with iodine to form an iodine complex in the following way,  $I_2 + I^- \rightarrow I_3^-$  and it can be shown both theoretically and experimentally that greater quantities of this complex are present in neutral than in acid solution. This suggests the possibility that it may be the  $I_3^-$  which is responsible for growth. If this is true, then it would be expected that a solution containing a high concentration of  $I_3^-$  should induce growth even at the high hydrogen ion concentrations at which little growth is obtained by means of iodine alone.

A brief experiment utilizing the starch test for iodine seems to indicate that  $I_3^-$  is formed in highest concentrations at the neutral points. The reaction of starch with iodine to form a blue starch-iodine compound has been shown by Mylius (1887) to be favored by the presence of iodide ions. It is therefore thought that it is  $I_3^-$  which gives the starch-iodine test rather than free iodine. An experiment was performed to test roughly at which hydrogen ion concentration one would expect to find the greatest starch-iodine reactions; *i.e.*, greatest production of  $I_3^-$ . It was found that buffer solutions containing small quanti-

ties of iodine and starch at pH 4.3, 5.8, 7.0, and 8.6 produce the violet color only at pH 7.0. At pH 8.6 the solution is colorless, at pH 5.8 it is amber (with a faint blue), and at pH 4.3 it is amber. Thus  $I_3^-$  seems to be produced maximally at about the neutral point and this would support the hypothesis that  $I_3^-$  is responsible for the induction of growth.

As for the theoretical viewpoint, if we consider equation (5) to be the only reaction occurring and, from the equilibrium reaction, calculate the amount of iodide formed at pH 4.3 and at pH 7.0, we find that 50 per cent of the iodine which does react according to this equation is converted to iodide at pH 7.0 and only 3 per cent at pH 4.3.

$$\frac{[IOH] \times [H^+] \times [I^-]}{[I_2]} = 3 \times 10^{-13}^*$$

\* Ephraim (1934).

Thus the iodine which actually does enter into the reaction would be expected to yield more iodide and consequently more  $I_3^-$  at pH 7.0 than at pH 4.3. An experiment was performed to test whether or not tadpoles raised under these different conditions exhibit any diverse effects which may be attributed to the different concentrations of  $I_3^-$ .

#### (a) Procedure

Two groups of twelve tadpoles were procured on Mar. 28, 1941. One group was kept in a solution of elemental iodine, the other in a mixed solution of potassium iodide and elemental iodine. The final iodine concentration in both solutions amounted to  $6.25 \times 10^{-6} M$  and the hydrogen ion concentration was adjusted to pH 5.2. Two other groups of eight tadpoles each were used in a parallel experiment in which the hydrogen ion concentration was adjusted to pH 6.8.

#### (b) Results

After 26 days, the average growth of the animals amounted to 2.2 mm. in the iodine solution and 2.0 mm. in the iodine-complex solution. This difference is not statistically significant and indicates that the iodine-complex is not more potent as an inducing substance than iodine. This experiment therefore fails to confirm the hypothesis advanced above to account for the difference in inducing ability of iodine solutions at different hydrogen ion concentrations.

The results of the second experiment add little. In the same period of time (26 days) these animals in both types of solutions, although smaller than those used at pH 5.2 grew more rapidly. They averaged an increased growth of 3.1 mm. in the iodine and 3.0 mm. in the iodine-complex solutions. Thus the inducing capacities of the iodine-complex and the elemental iodine solutions are seemingly similarly affected by a change in hydrogen ion concentration from pH 5.2 to pH 6.8.

## IV. DISCUSSION

The results which have been obtained in this investigation seem to present a rather complex situation the solution of which cannot yet be said to be complete. Nevertheless, certain facts have been ascertained which add to our knowledge of the factors involved in the problem under investigation and which will undoubtedly be of importance in the final solution of the matter.

Rosen's finding, that the induction of metamorphosis by means of thyroxin dissolved in the environmental solution is influenced by the hydrogen ion concentration of the solution, has been definitely confirmed. With the same thyroxin concentration, animals kept in solutions of higher acidity show a greater response than do those kept in solutions of lower acidity. Thus as Rosen pointed out, acidity accelerates and alkalinity retards the metamorphosing action of thyroxin.

Several hypotheses can be advanced to explain these results. The potency of thyroxin itself may conceivably be affected by a change in hydrogen ion concentration, so that it would be more effective in acid solutions. However, our experiments with injected thyroxin (Section A) seem to make such an explanation untenable, for hydrogen ion concentration does not affect the inducing power of thyroxin when the thyroxin is injected into the animal. This being so one must conclude that the hydrogen ion concentration of the environment does not act directly upon the thyroxin molecule itself, decreasing its effectiveness as an inducing agent, but that instead the hydrogen ion concentration of the environmental solution brings about in some way an effect on the availability of dissolved thyroxin to the animal. We know that this cannot be due to a solubility effect because thyroxin, being more soluble in alkaline solutions than in acid solutions would hardly be expected to supply more thyroxin to the tadpole in acid solution. Possibly the effect could be one which acts upon the integument of the animal in such a way that the permeability of the skin to thyroxin is greater at high hydrogen ion concentrations than at low. We have not investigated this possibility, but feel that the results of our experiments with the effects of hydrogen ion concentration upon the respiratory metabolism of the tadpole strongly indicate the method by which the tadpole is furnished more thyroxin from a more acid environment.

The water of the environment furnishes the source of water and salts to the tadpole. Since the animal does not drink water, dissolved thyroxin enters the animal through the integument and the gills and the amount of thyroxin absorption might be expected to be dependent on the rate at which water enters the animal as well as on the amount of dissolved thyroxin. The present experiments clearly show that the rate of respiration is increased in acid solution and decreased in alkaline solution. An increased rate of metabolism may be thought to result in an increased intake of the environmental fluid and a reduced rate of metabolism to result in a reduced intake. Thus, in acid en-

vironments the tadpole takes up dissolved thyroxin more rapidly than it does in alkaline environments. Under these conditions, a more rapid growth of the hind limbs would be expected in tadpoles living in acid solutions than would be expected in those living in more alkaline environments.

Iodine-induced development is quite different from thyroxin-induced development and would seem to be more difficult to interpret. In thyroxin induced development, as we have seen, there appears to be no effect of hydrogen ion concentration upon the molecule itself. However, such an effect does seem to occur in iodine-induced development and it is this chemical effect which is of prime importance here. The existence of a chemical effect is demonstrated by experiments in which the effects of hydrogen ion concentration on induced growth are the same regardless of whether the iodine is injected into the animal or enters from the environment. In other words, in this case it is not a question of how much iodine enters the animal, but at the hydrogen ion concentration and consequently in what form. The efficiency of iodine as an inducing agent is at a maximum at about the neutral point. This is true whether the animal is injected with iodine at this hydrogen ion concentration or the environmental solution contains iodine at this hydrogen ion concentration. There is then a marked lowering of effectiveness as the hydrogen ion concentration is either increased or decreased from this point.

Several experimental attempts have been made to ascertain what chemical substance or substances, produced by iodine at the neutral point may account for its effectiveness at this hydrogen ion concentration. The idea was advanced that the effectiveness of iodine at the neutral point is due to the presence of some form or forms of iodine at this hydrogen ion concentration other than free iodine alone. We have reason to believe that free iodine of itself is not a powerful inducing agent, for increased acidification of an iodine solution results in increased quantities of free iodine but decreased growth.

At different hydrogen ion concentrations, iodine solutions contain various quantities of iodide, iodate, and iodite as was evident from the several equations presented in Section I. Iodate is absolutely ineffective as an inducing agent when used alone. Iodide has but a slight effect when used alone and iodite is presumably ineffective, for the effect of increasing the iodite concentration at the expense of free iodine is only to reduce the efficiency of the iodine solution as an inducing agent.

It has been pointed out that free iodine reacts with iodide to form the iodine complex  $I_3^-$ , and that an iodine solution contains a maximum concentration of  $I_3^-$  at precisely the same hydrogen ion concentration as that which affords maximum growth. Experimentation indicated, however, that this is merely a coincidence, for solutions containing  $I_3^-$  are no more efficient in inducing growth when in acid solutions than are solutions of elemental iodine.

It is therefore not possible to say what it is which is produced at the neutral point which makes iodine an effective inducing agent at that hydrogen ion concentration. The results which have been obtained and the explanations given point the way for future experiments which must be performed before this aspect of the problem can be solved.

A study of the effects of hydrogen ion concentration on normal development proved to be of interest. This effect paralleled the effect of hydrogen ion concentration on thyroxin-induced development. Acidity accelerates the development. This can be accounted for if *Elodea*, which is used in the feeding of the animals in their normal development, contains iodine organically bound and therefore, like thyroxin, may be unaffected by hydrogen ion concentration. The differential growth effect would then be attributable, as in the case of thyroxin-induced development, to the differential effect of hydrogen ion concentration on the rate of metabolism of the animal.

Throughout the different experiments it has been evident that there is a marked difference in the variability of response of the tadpoles. This is interesting enough to warrant further attention. The variability of response of the animals is seemingly dependent upon the strength of the environmental stimuli. Substances, like thyroxin, which are very potent inducing agents produce a less varied response than less potent substances like iodine or potassium iodide. And these latter, in turn produce a less varied response than that which is found in normal development. Substances which are responsible for a particular process then, such as the metamorphosis of the tadpole, bring about this process with less variability as the effectiveness of this substance is increased either by concentration or potency. A rigid environment, such as is obtained by the use of buffer solutions also makes for a less variable response of the animals. We saw this in the respiration experiment. Here, variability of response was much greater when animals were not kept in buffer solutions.

#### V. SUMMARY

1. It has been shown quantitatively that the degree of response of the hind limbs of tadpoles to the action of thyroxin is dependent upon the lengths of the limbs at the beginning of treatment.
2. Both the potency of the inducing substance and the rate of penetration of the substance into the animal might be involved in the effects of hydrogen ion concentration on induced development.
3. Changes in hydrogen ion concentration affect the inducing power of thyroxin and iodine differently. With thyroxin, it is the rate of penetration of the molecule which determines the amount of growth, but with iodine it is the chemical form in which the substance has entered the animal which is of prime importance.

4. The hydrogen ion concentration of thyroxin solutions does not affect their potency when they are injected into tadpoles.

5. Change in hydrogen ion concentration of the environment does not affect the potency of thyroxin injected into tadpoles.

6. When thyroxin is administered in the environmental solution its effects, as measured by increase in hind limb length are greater at higher than at lower hydrogen ion concentrations in the range tested.

7. Since the potency of thyroxin is unaffected by change in hydrogen ion concentration when the thyroxin solution is injected, the above fact (point 6) seems explicable only on the basis of differences in the rate of penetration of thyroxin into the animals at the different hydrogen ion concentrations.

8. These differences in penetration of the thyroxin at different hydrogen ion concentrations may be the result of a differential effect of hydrogen ion concentration upon the rate of metabolism of the animal. The metabolic rate is significantly greater when the tadpoles are kept in solutions of higher hydrogen ion concentration than when they are kept in solutions of low hydrogen ion concentration. It is postulated that the rate of metabolism, since it controls the rate of intake of the environmental fluid and therefore of dissolved thyroxin, also controls the amount of thyroxin-induced development.

9. Change in hydrogen ion concentration of iodine solutions affects their potency when injected into tadpoles. A peak of effectiveness is reached at about the neutral point, with a lowered efficiency as the hydrogen ion concentration is either increased or decreased from this point.

10. Change in hydrogen ion concentration of the environment affects the potency of iodine injected into tadpoles. The effect is similar to that noted in point 9.

11. The hydrogen ion concentration of the environment seems to affect the chemical nature of the iodine in solution in the environment. If this is so, it is possible that the differences in the metamorphic effects of iodine at different hydrogen ion concentrations are dependent upon the chemical form of iodine present.

12. The effect of hydrogen ion concentration on normal development is similar to that on thyroxin-induced development; an effect on the rate of metabolism of the animal causes increased growth in more acid solutions.

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# CERTAIN ENZYMATIC ACTIVITIES OF NORMAL AND MOSAIC INFECTED TOBACCO PLANTS

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We have described in a previous paper (Wynd) the disturbances in the respiratory activities, as exhibited in the rates of oxygen use, which tobacco plants undergo during the course of general physiological changes following infection with the leaf mosaic virus. The present paper describes the changes occurring in the activities of oxygenase, peroxidase, catalase, and invertase.

The plants studied were the same individuals which furnished the data on the rates of oxygen use. The previous paper should be consulted for the details of the experimental arrangement. Briefly, Burley tobacco plants were transplanted from flats into small pots, and successively transferred to larger pots as growth proceeded, until they were in 8 inch pots. When the plants had five well developed leaves, the two lower were removed and the lowest remaining leaf was then inoculated with a virulent juice pressed from the leaves of plants showing severe symptoms of the mosaic disease. This lower, inoculated leaf was designated as No. 1, and successively higher leaves numbered in sequence. As new leaves appeared and became large enough for study, they were designated numerically in the proper sequence.

Three normal and three inoculated plants were moved to the laboratory for study at successive intervals of 2 or 3 days. After the excision of the discs for the observation of the respiratory rates described in the previous paper, the similarly numbered leaves were removed from the plants and finely ground with purified quartz sand in a porcelain motor and the juice extracted by squeezing the pulp through cheese-cloth. The data, therefore, are based in each instance on a composite sample of comparable leaves from three plants.

The exigencies of the experimental procedure which especially demanded a great many separate procedures in duplicate to be carried out rapidly and simultaneously on aliquots of the expressed juice, unfortunately eliminated the possibility of obtaining duplicate series of determinations on different groups of plants. Even though the juice represented a composite sample from three leaves of similar age and stage of development, there is always the possibility that a single abnormal plant could give a fictitious value to the determination based on this sample. That this was not likely, however, is suggested by a comparison of all the curves which shows that the trends which

they exhibit are dependent on determinations made on successive days on separate groups of plants. And especially does this seem improbable when we notice the instances in which a period of maximum or minimum activity of an enzyme does not occur simultaneously in all the leaves of the plant. For example, the period of the second maximum in the activity of invertase described below occurs in all leaves but at a progressively earlier period in the younger leaves, and hence it could not be an error dependent on single plants reacting atypically.

Data are presented as ratios of the activity of inoculated plants to that of the normal plants. The present study is not concerned with the physiology of the separate enzymes, and their activities are followed only as an index to physiological disturbances. Since this is the case, the ratio of the normal plants to that of the inoculated plants becomes the logical value basis for comparison. Further, daily changes in greenhouse conditions and in laboratory temperature lessen the significance of an absolute value of an enzymatic activity.

#### *Oxygenase and Peroxidase*

The earlier literature concerning the oxidizing enzymes frequently uses the term "oxidase" with a vaguely defined meaning. Strictly speaking, this term should be replaced by oxygenase and peroxidase. Oxygenase is to be considered as the enzyme catalyzing the oxidation of certain polyphenolic substances by molecular oxygen with the formation of quinones and peroxides. The magnitude of the reaction may be detected directly by measuring the volume of oxygen used by appropriate manometric means or indirectly by determining the amount of pigment developed by these quinones. Peroxidase is determined by determining gravimetrically or colorimetrically the amount of pigment developed from the colorless dye reagent in the presence of a few drops of hydrogen peroxide. The terms appearing in the earlier literature have been modified to conform with the above definition.

Woods (1899, 1900, 1902) found that oxygenase and peroxidase were more active in the leaves of tobacco plants infected with the leaf mosaic disease than in the leaves of normal plants. He attached considerable importance to this condition because the mere injection of preparations of these enzymes produced symptoms of the disease under some conditions. He further observed that these enzymes had the power to inhibit diastase *in vitro* and concluded that this might be the mechanism of the effect of the increased oxidizing enzymes that accompanied the appearance of the disease or were injected artificially.

Similar observations were made by Suzuki (1902*a*) who reported that the mulberry dwarf disease, a virus disease widely spread in Japan, accompanied an increase of 400 to 500 per cent in the activity of oxygenase and 200 to 500 per cent in the peroxidase. The above observations were made on leaves collected in the autumn but similar results (1902*b*) were obtained with material collected during the spring.

Suzuki (1902*c*) in a more detailed paper reported that this excess of the oxidizing enzymes was accompanied by a lower nitrogen content. Shibata (1903) also found greater oxygenase activity in mulberry leaves infected with virus.

Virus-infected cabbage leaves were found to have more oxidizing power by Hasselbring and Albsberg (1910) although these authors attributed this to a decrease in anti-oxidase rather than to an increase in the oxidizing enzymes themselves.

Pantanelli (1912) found that virus-infected grape leaves exhibited greater oxygenase and peroxidase activity and he believed that this stimulated activity caused an increased production of carbon dioxide.

Bunzel (1912 *a, b*, and 1913 *a, b*) studied the curly top disease of the sugar beet and observed an increase of 200 to 300 per cent in the oxygenase activity. This increase was observed in plants which had been stunted in their growth by such agencies as excess watering, drought, and by various agencies of unknown nature. A later publication by this author (1914) reported an extensive study of the oxidizing enzymes in healthy and curly dwarf potatoes. Normal plants showed a diminishing oxygenase activity during the 60 to 80 days of active growth, but an increased activity occurred during the normal cessation of growth. Possibly the same physiological factors cause this increase as operate during the slowing of growth due to disease.

Chapman (1913) found an increased oxygenase and peroxidase in mosaic-infected leaves of tobacco and tomato and considered this to be a cause of the disease. Freiberg (1917) also found an increased oxidizing activity but considered this to be the result rather than the cause of the disease.

An increased oxygenase activity was found in virus-blighted spinach by Bunzell (1918). The leaf roll disease of potatoes was shown by Neger (1919) to be accompanied by increased oxygenase and peroxidase.

In spite of the reports of the above authors an increased oxygenase and peroxidase activity has not been found always to accompany virus infection. For instance, Hunger as early as 1903 (1903, 1905) doubted if differences between healthy and mosaic tobacco were significant. Doby (1911 *a, b*) found that both normal and healthy potato tubers showed very erratic oxidizing activities and finally decided that virus infection could not be detected by observing the action of these enzymes.

The activities of oxygenase and peroxidase were determined in the present work by the method of Guthrie (1931). This author points out that the mixture of alpha-naphthol and paraphenylenediamine is oxidized far too rapidly for quantitative determination of oxygenase and peroxidase if the mixture is near neutrality, but if the reacting solution be buffered at pH 4.5, the rate of oxidation is sufficiently slow to allow accurate comparisons to be made. The acidity also inhibits catalase and prevents the interference of this enzyme.

A stock solution of a citrate buffer at pH 3.5 was prepared by dissolving 21 gm. of citric acid in 170 cc. of 1*N* sodium hydroxide and diluting to 1 liter. This solution may be preserved for several days in the refrigerator. The reduced dye reagent used to detect the extent of the enzymatic oxidation was prepared fresh just before

use. The substrate was prepared by adding 200 cc. of the citrate buffer to 200 cc. of water and then adding 1 gm. of paraphenylenediamine hydrochloride. To this solution were added 20 cc. of 4 per cent alpha-naphthol in 50 per cent ethyl alcohol. This was filtered and used immediately. There is almost no autoxidation of this substrate at the indicated pH. Aliquots of 50 cc. were added to a series of large test tubes. For the oxygenase test, 1 cc. of the expressed juice of the leaf tissue was added. For the peroxidase test, 0.3 cc. of juice and 0.2 cc. of commercial hydrogen peroxide were added. After standing, with occasional shaking, at room temperature for 10 to 20 minutes, the reaction was stopped by adding 5 cc. of 0.1 per cent potassium cyanide. Then 25 cc. of toluene were added, the tubes stoppered, and vigorously shaken until the toluene layer had extracted all of the color from the aqueous layer. The supernatant colored layer was poured into a tube, stoppered, and briskly centrifuged for 2 or 3 minutes. The color of the toluene extract from leaves of inoculated plants was compared to that from corresponding normal leaves.

The changes in the activity of oxygenase of the leaves from inoculated plants relative to that of corresponding leaves of normal plants are assembled in Table I and are graphically represented in Fig. 1. At the time that leaf 1 was inoculated, only four well developed leaves were present on the plant. Each of these leaves exhibits a diminishing oxygenase activity, and reached a minimum on the 4th to the 6th day. The fifth leaf was large enough to be included in the observations on the 6th day and this leaf reached its minimum at the 10th to the 12th day. The sixth leaf was included in the study on the 11th day and its minimum was reached on the 14th day. All leaves, therefore, are seen to go through a preliminary period of lessened oxygenase activity relative to normal leaves and the leaves appearing later on the plant show the lowest point of this activity on a progressively later date. Further, all leaves from the inoculated plants show a comparatively abrupt increase with a maximum at about the 14th day, and following this, the activity diminishes until it is below that of normal leaves in most cases. In general, these relationships are more pronounced in the lower leaves of the plant.

The data reported in the previous paper (Wynd) show that these plants contained detectable amounts of the virus in all their leaves on about the 14th day which coincides approximately to the time of maximum oxygenase activity.

The theory of Woods (1902) that the increased oxygenase activity interfered with normal metabolism of the leaf and thereby produced the visible symptoms of the disease is partially substantiated by the present data. A serious objection, however, lies in the fact that the activity of the enzyme so quickly lessens once its maximum is reached, until it approaches, or falls below, that of normal plants.

The changes in the activities of peroxidase are not so well marked as those of oxygenase and we have not observed increases of the same order of magnitude as have been reported by other workers. In all experiments, a vigorous peroxi-

TABLE I

*The Oxygenase Activities of the Leaves of Tobacco Plants Inoculated with Leaf Mosaic Virus Expressed As Percentages of the Activity of Corresponding Leaves from Normal Plants*

Days after inoculation	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6
2	84	137	93	116	—	—
4	77	66	81	80	—	—
6	49	75	91	100	100	—
8	99	103	95	100	72	—
11	167	129	143	125	80	125
14	200	250	100	100	200	100
16	116	125	165	86	38	125
18	111	111	125	83	167	100
21	113	116	135	83	42	113

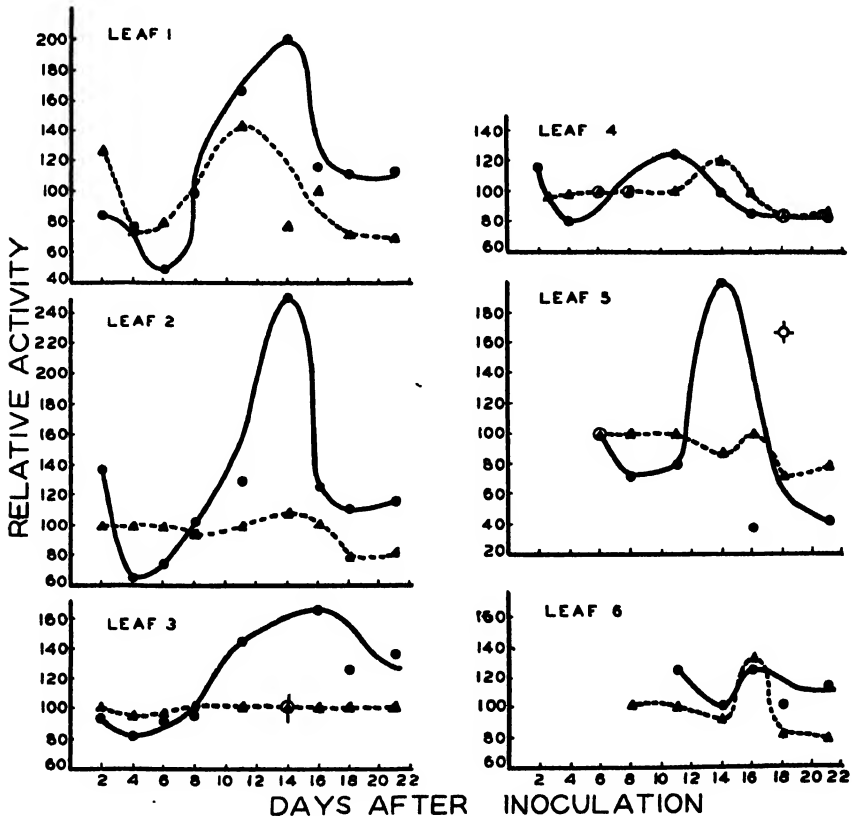


FIG. 1. The activities of oxygenase (solid line) and of peroxidase (dotted line) of virus-infected tobacco leaves relative to their activities in normal leaves.

dase reaction was obtained which would eliminate the possibility that an error had occurred in preparing the reagents.

An examination of the data in Table II and the graphs in Fig. 1 shows that only in leaves 1, 4, and 6 was there even a qualitative relation between the changes in peroxidase and oxygenase. In the later stages of the disease, there is a definite diminution in the activity of this enzyme in diseased leaves as compared with that in normal leaves of the same age. The author believes that recent developments in the chemistry of cellular oxidation show that these enzymes need not be correlated necessarily qualitatively or quantitatively. A discussion of these developments in cellular oxidation will be presented in a future paper.

TABLE II

*The Peroxidase Activities of the Leaves of Tobacco Plants Inoculated with Leaf Mosaic Virus Expressed as Percentages of the Activities of Corresponding Leaves from Normal Plants*

Days after inoculation	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6
2	127	100	100	98	—	—
4	74	100	94	98	—	—
6	80	100	95	100	100	—
8	100	95	100	100	100	100
11	143	100	100	100	100	100
14	77	108	100	120	87	91
16	100	101	100	100	100	131
18	71	79	100	83	71	80
21	69	81	100	83	78	78

### *Catalase*

Suzuki (1902 *a*) reported that an increased catalase activity accompanied the mulberry dwarf disease in Japan and he made the doubtful conclusion that this increase was physiologically effective in destroying the hydrogen peroxide that developed in the tissues as a result of the high rate of respiration. In a later study (1902 *b*), he was unable to detect any definite trend of the activity of this enzyme although the data vaguely indicated that a little greater catalase activity probably existed in the diseased leaves. Chapman (1913) found that catalase activity was greatly diminished in both tobacco and tomato leaves when they were infected with the mosaic virus.

The comparative activities of the catalase from various leaves were determined in the present study by adding 1 cc. of the press juice to a mixture containing 25 cc. of water, 25 cc. of a phosphate buffer at pH 7.0, and 10 cc. of commercial 3 per cent hydrogen peroxide. The reaction proceeded for 1 hour, with constant agitation on a mechanical shaker. The reaction was stopped by adding 25 cc. of a 25 per cent

solution of sulfuric acid. The amount of hydrogen peroxide destroyed by the enzyme was determined by titrating the unused peroxide with 0.1 N potassium permanganate.

The data are presented in Table III and Fig. 2. It is difficult to detect any definite trend in the changes in catalase activity. The method of determination is capable of considerable accuracy and the apparently erratic values are not due to errors in the experimental procedure. The lower three leaves were well developed at the time that the lower leaf was inoculated. These leaves of the inoculated plants appear to have a catalase activity near that of normal leaves for the first few days, but by the 6th day, it surpasses that of the normal leaves. A new low is reached, however, after about 11 to 14 days. A small subsequent rise appears at about the 18th day. These three comparatively mature leaves thus appear to react similarly.

TABLE III

*The Catalase Activities of the Leaves of Tobacco Plants Inoculated with Leaf Mosaic Virus Expressed as Percentages of the Activities of Corresponding Leaves from Normal Plants*

Days after inoculation	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6
2	69	92	86	105	—	—
4	113	97	126	127	—	—
6	105	157	135	131	76	—
8	204	315	223	67	60	47
11	210	92	154	111	81	100
14	138	216	130	96	125	92
16	105	254	131	135	140	129
18	170	198	158	169	354	146
21	160	607	149	117	124	90

The fourth leaf was very small at the time of inoculation and it is seen that this leaf, like leaves 5 and 6 which subsequently appeared, shows the lowest activity on about the 8th day, or about 10 days before the minimum appeared in the older leaves. It is especially interesting to notice that these three younger leaves show very similar curves of their catalase activity, differing only in that the earlier changes are omitted from the data since the leaves concerned were not yet large enough for study. This group of young leaves thus is seen to react similarly, and differ from the group of mature leaves chiefly in that the preliminary period of increased activity is very much smaller in magnitude (see leaf 4) and progressively disappears (see leaves 5 and 6), and also in the fact that the period of minimum activity appears about 10 days earlier.

It is difficult to attach a physiological significance to the sequence of changes that occur in the catalase activities of these leaves from inoculated plants. We can do no more than suggest that the period of minimum activity in the



three older leaves, which were present when leaf 1 was inoculated, appeared at about the time that they attained the ability to infect young plants with the virus, and that the younger leaves became infectious earlier, which again

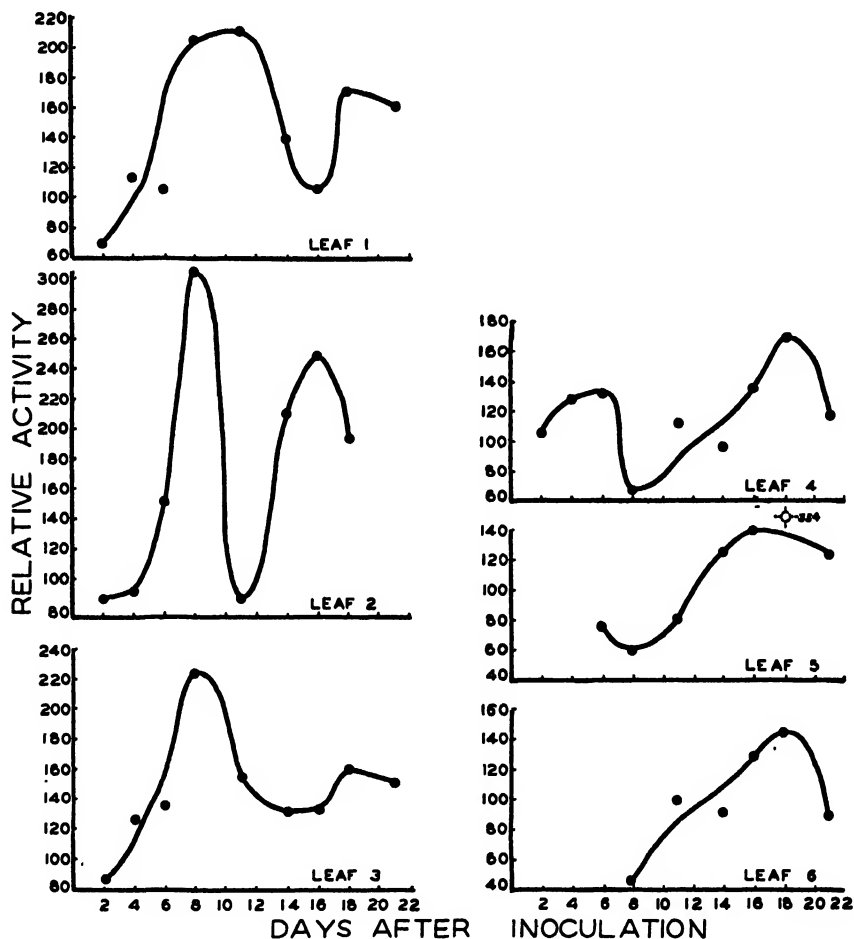


FIG. 2. The activity of catalase of virus-infected tobacco leaves relative to normal leaves.

approximately corresponds to the time that a detectable amount of virus appears.

In view of the sequence of changes in the catalase activity which follows infection of the tobacco plant, it appears that the apparently erratic values which Suzuki obtained for mulberry leaves also could have been due to differences in leaf development or to the particular stage of the disease at the time

of observation. The reduced catalase activity reported by Chapman also appears to be dependent on the stage which his plants had attained.

A comparison of the changes in the catalase activities with those of respiration rates previously reported (Wynd) shows that the maximum rate of oxygen use occurred at about the 4th to the 6th day when the catalase was at its minimum. Consequently Suzuki's belief that the increase in catalase was physiologically significant because it destroyed the excess of hydrogen peroxide produced by the augmented respiration seems very improbable.

### *Invertase*

The author has been able to find but a single reference in the literature concerning the invertase activity of plants infected with a virus. This is particularly strange in view of the large amount of attention that has been given to the carbohydrate metabolism of plants affected with these diseases. Schaffnit and Ludtke (1930) were unable to detect a definite change in the invertase activity of tobacco plants infected with mosaic.

The activity of the invertase in the juice prepared from the tobacco leaves as described above was determined by adding 1 cc. of the juice to 50 cc. of a 10 per cent sucrose solution and incubating at 37° C. for 72 hours. Bacterial action was prevented by adding 2 cc. of toluene to each flask. The flasks were stoppered to prevent the loss of the toluene during the period of incubation. Aliquots of 10 cc. of the incubated mixture were added to 50 cc. of Fehling's solution and brought to boiling in 3 minutes. After boiling for 2 minutes, the mixture was quickly filtered. The residue was dissolved on the filter by washing with 50 cc. of Bertrand's reagent consisting of 20 per cent sulfuric acid saturated with ferric sulfate. The amount of iron reduced by the cuprous oxide was titrated with 0.1 N potassium permanganate. Determinations were run on the amount of reduction brought about by aliquots of fresh juice and also on the amount of sugar hydrolysis that occurred during the period of incubation in the absence of the enzyme and the appropriate corrections applied.

The data are assembled in Table IV and are presented graphically in Fig. 3. It is evident that the lower three leaves, all of which were well developed when the lowest leaf was inoculated, reacted similarly. There was a preliminary rise in the invertase activity which reached a maximum on about the 4th day after leaf 1 had been inoculated. This was soon followed by a depressed activity, which reached a minimum on about the 8th day. This was followed by a second rise in invertase activity which reached a maximum at about the 14th to the 16th day. It is seen that the magnitude of this second maximum becomes progressively less in the leaves higher on the stem. It was at this time that these leaves attained the power to infect young tobacco plants. Leaf 1 naturally was infectious from the first, since it had received the original inoculation. It does not follow, however, that leaf 1 had developed new virus

TABLE IV

*The Invertase Activities of the Leaves of Tobacco Plants Inoculated with Leaf Mosaic Virus Expressed as Percentages of the Activities of Corresponding Leaves from Normal Plants*

Days after inoculation	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6
2	124	103	100	90	—	—
4	137	130	118	122	—	—
6	78	105	106	85	91	—
8	124	72	77	92	100	109
11	105	143	110	106	103	117
14	122	91	109	98	98	92
16	273	145	94	72	75	65
18	175	143	78	81	74	91
21	131	89	109	94	95	91

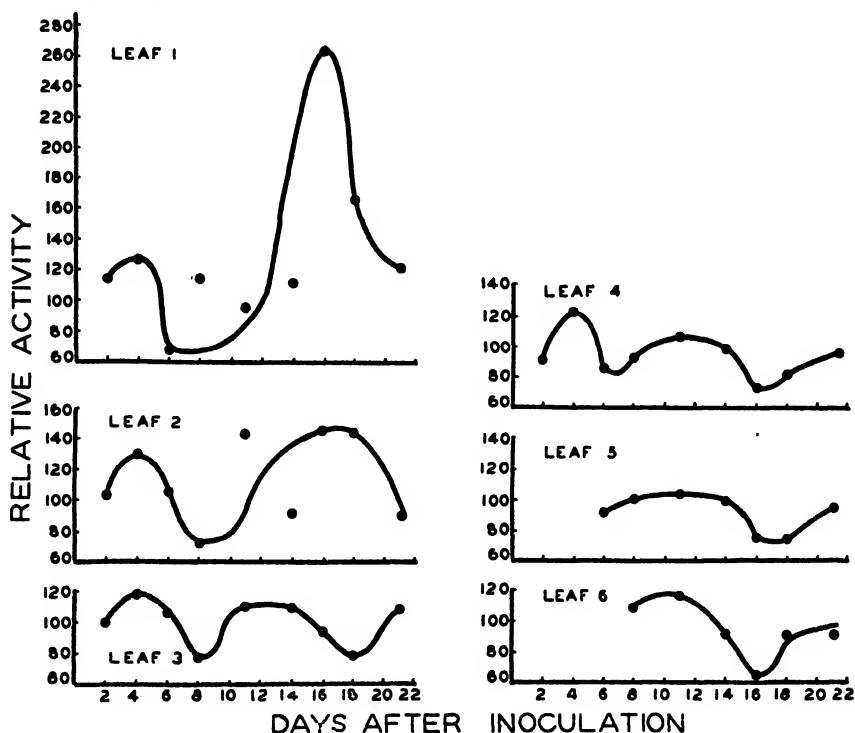


FIG. 3. The activity of invertase of mosaic-infected tobacco leaves relative to normal leaves.

before this time since a remainder of the original inoculated material could have infected the test plants. Following the second maximum, there was a

decrease until the activity of invertase approached or became less than that of the normal plants.

When the lowest leaf was inoculated, the fourth leaf was very small and leaves 5 and 6 were rudiments only, and it may be seen that these three younger leaves react similarly. Like the older group of leaves, leaf 4 exhibits a preliminary maximum on about the 4th day, which was followed by a minimum at about the 6th to the 8th day. Leaves 5 and 6 were not large enough to be studied until the 6th and 8th day respectively, and consequently the period of the first maximum and minimum were lost, but the trend of their curves in each case seems to indicate a recovery from a minimum occurring as in the older leaves, probably on about the 6th to the 8th day. A rise to a second maximum occurs in each of the younger leaves as was also true of the older leaves but the magnitude of this maximum was much less and occurred on about the 10th to the 12th day which was about 6 days earlier than in the older leaves. This second maximum was followed by a second period of depressed invertase activity, as in the older leaves, but differing in that the minimum was considerably lower and occurred about 6 days earlier. When the oldest leaf was at the period of its second maximum, on about the 16th day, the younger leaves had already passed this period and were in the period of their second minimum. A comparison of all the curves in Fig. 3 shows that the period of the second maximum appears progressively earlier in the younger leaves. For instance, leaf 1, the oldest leaf, reached this stage on about the 16th day, the second leaf about the 14th, the third about the 13th, and the fourth and all younger leaves on about the 11th day. A further significant difference occurs in the younger leaves. The second minimum is followed by an upward approach to a normal invertase activity. Since the observations continued for only 21 days, it is not known whether the older leaves might also exhibit this recovery at some later time.

#### SUMMARY

The lower leaves of tobacco plants were inoculated with leaf mosaic virus and the activities of oxygenase, peroxidase, catalase, and invertase were followed in leaves of comparable age at intervals of 2 or 3 days over a period of 21 days.

The inoculated leaves exhibited a great decrease relative to normal tissue in the activity of oxygenase and peroxidase on the 6th day. Younger leaves showed this minimum at a progressively later date. A great decrease in the activities of these enzymes was attained by the 14th to the 18th day. This maximum was followed by a decrease.

Catalase exhibited an increased activity which reached a maximum at about the 8th day. A second maximum was observed on the 16th to the 18th day.

Invertase reached a minimum, relative to normal plants, on about the 8th day. A second minimum was approached on the 16th to the 18th day.

These data show that profound disturbances in the physiology of infected plants occur many days before the leaf juice attains an infectious concentration of virus. The observed activities could not be due therefore to metabolic activities of the virus particles themselves.

Since infectivity is attained only after a period of profound physiological disturbance, it seems possible that the virus protein develops as a product of abnormal metabolism.

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# A SIMPLIFIED DIFFERENTIAL MICRORESPIROMETER\*

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## INTRODUCTION

Cunningham and Kirk (1) described a differential microrespirometer constructed of metal, which had a number of advantages over previously described instruments. Most important of these was its freedom from the necessity of maintaining a constant temperature by means of a thermostat. In addition, it was highly sensitive (depending on the capillary diameter, down to  $\pm 0.001\lambda$  reading accuracy); it permitted the use of any desired gas mixture; it allowed mixing of solutions during a run; and it could be adapted to numerous types of biological material. Its most important deficiency was the difficulty of construction to render it gas tight. Because of the large amount of labor necessary, the cost of multiple units became relatively high.

The recently described respirometer of Tyler and Berg (2) also avoids the necessity of maintaining a constant temperature. It is subject to errors due to uneven heating of its two gas spaces and its construction from glass does not facilitate rapid equilibration of these spaces when unsymmetrical temperature changes occur. It is customarily operated in a water bath for this reason and to keep the organism at a known temperature. Only when the latter precaution is necessary, is there any reason for keeping the metal differential instrument in a constant temperature bath.

In order to simplify the construction and reduce the cost to a point which was practical for routine and class use, the instrument described here was developed as a modification of the original instrument of Cunningham and Kirk. It offers nearly all of the advantages of the original instrument combined with simple, inexpensive construction and greater adaptability to multiple determinations.

## *Description*

The respirometer consisted of a rectangular metal block containing two identical chambers, covered with a head plate of the plastic, lucite (or metal), on top of which was placed a capillary carrying an index droplet of kerosene.

\* Aided by grants from The Rockefeller Foundation and the Research Board of the University of California.



The apparatus was held together by means of a strip of channel brass on top of the capillary, the strip being attached by means of screws to the block. Seals were effected throughout by means of stopcock grease and rubber washers.

A detailed construction diagram is shown in Fig. 1. The main block, *A*, was constructed from a 3 inch length of rectangular brass,  $1\frac{1}{4} \times \frac{3}{4}$  inches. One narrow face was machined to form a rim around each of two chambers,

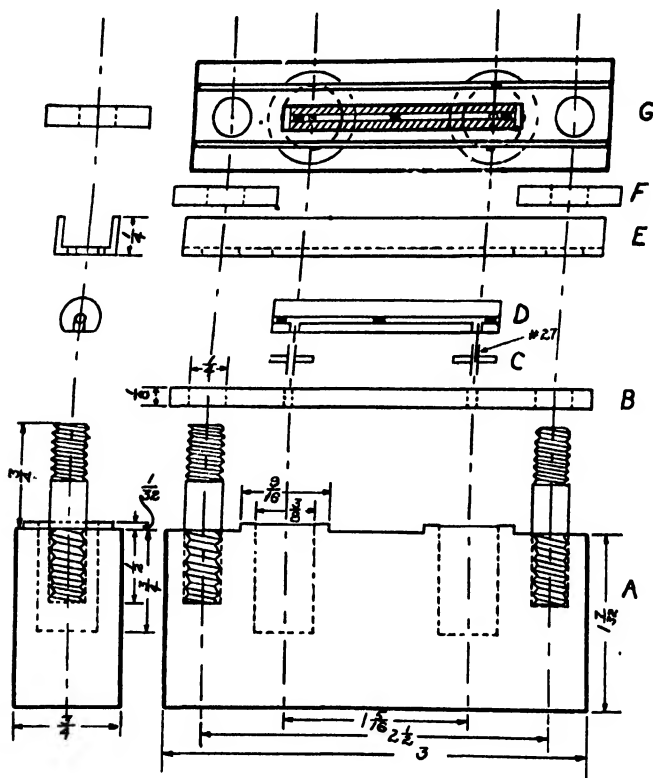


FIG. 1. Construction diagram of differential microrespirometer.

which were drilled symmetrically  $1\frac{5}{16}$  inches apart and  $\frac{3}{8}$  inch in diameter. At each end of the block was inserted a threaded bolt which served to fasten the apparatus together. On the block was fitted a head plate *B*, of the same dimensions as the top of the block and consisting of a flat piece of lucite,  $\frac{1}{8}$  inch thick (or a similar brass piece), drilled to slide over the end bolts and with a 1 mm. hole drilled over each chamber center. Head plates made of lucite had the advantages over metal plates of allowing observation of the organism during a run, and of a direct check on the continuity of the grease seal to the chamber block.

Above the head plate was placed a glass capillary tube *D*, prepared as follows: A capillary tube of appropriate and uniform diameter (0.1 to 1.0 mm.) and 1 3/4 inches in length was flattened on one side by grinding to a width of 1/8 to 5/16 inch. The surface was polished somewhat to remove frosting, by means of a smooth stone. Two holes were drilled through the flat side to connect with the capillary bore and separated by the distance between the chamber centers, 1 5/16 inches. A blunt 27 gauge syringe needle was used with grinding compound to drill these holes. To insure a tight fit of the capillary to the head plate, two small rubber gaskets *C*, were used, each of them perforated by a short length of 27 gauge hypodermic needle. The capillary was carefully cleaned with chromic acid mixture to insure smooth movement of the index droplet. The ends of the capillary were stoppered with small plugs of paraffin which were pushed into place and sealed by mild warming.

The head plate and capillary were held tightly to the chamber block by means of a piece of channel brass strip *E*, drilled to slip over the threaded bolts attached to the block. The bottom of the channel brass was slotted over the entire length of the capillary *D*, so as to press only on the sides of the latter. The strip *E* was fastened down by two thin knurled nuts *F*, which served to tighten the connections of all the parts to give gas-tight seals. In clamping down the piece *E*, the screws had to be slowly and evenly tightened to prevent forcing the index droplet into the syringe needle connections. The position of the droplet was finally adjusted by slightly altering the tension of the thumb-screws, after which they were not disturbed for the duration of the measurements.

A scale cut from graph paper was placed under the center part of the capillary under the slotted portion of the channel brass piece, through which the movement of the index droplet could be read. The sealed surfaces were in all cases coated with stopcock grease applied with a small hypodermic syringe. These included the raised rim around the tops of the chambers, and both sides of the rubber gaskets used to seal the connections of the capillary to the head plate.

Alterations in the size of chambers were made by filling them to any desired height with paraffin or wax. The top surface of the material used was smoothed and adjusted approximately by use of a flat end of a metal rod of slightly smaller diameter than the chambers. It was necessary to fill each of the chambers to approximately the same height to obtain proper differential compensation. The actual volumes of the chambers after adjustment were in all cases determined by calibration with mercury, after greasing the metal to prevent amalgamation. Inside each chamber was placed a disk of lucite which carried a depression to hold the organism being studied and one for a droplet of caustic solution used to absorb the carbon dioxide produced by the organism. The volumes of these disks were also determined and used to cor-

rect the calibrated free space of the chambers. Small known differences in the free chamber volumes were used as corrections in making final calculations of the respiratory rate.

In use, the chambers were charged in exactly the same manner except for the presence of the organism in one, thus equalizing all effects except that being measured; *viz.*, the rate of utilization of oxygen.

The index droplet of kerosene, prepared as previously described (1), was placed in the capillary as follows: The tube was removed and turned upside down. A drop of kerosene was introduced into one of the drilled holes with a syringe needle. The excess was then removed by inserting the end of a hard-wood toothpick which served to suck up the kerosene slowly enough that rapid removal of the wood would leave any desired amount behind. This droplet was forced to the proper end of the scale and the capillary clamped in place.

After assembly of the apparatus, it could be tested for leaks by merely warming one end with the hand, which promptly forced the index droplet along the capillary. On removal of the hand, the droplet would soon return to the original reading if no leaks were present. No drifting of the droplet occurred when the apparatus was placed on a laboratory table for an indefinite time, exposed only to the normal temperature variations of the room.

A low power stereoscopic magnifier was found useful in reading the movement of the index droplet, since it made possible the easy determination of the meniscus position to 0.1 mm.

When the instrument was opened after a series of measurements, the kerosene index droplet frequently was lost into the hypodermic needle connections. It was removed by holding the opening of the head plate to a vacuum line. This also had the effect of keeping these fine openings free of obstruction.

#### EXPERIMENTAL

In order to test the operation of the respirometer described, the respiratory rates of numerous single pupae of *Drosophila melanogaster* were measured. The curves showing the oxygen consumption over a period up to 1.5 hours of three representative organisms, A, B, and C are given in Fig. 2. The details of these determinations are as follows: size of respiratory chamber, 189 $\lambda$ ; lucite cup, 64 $\lambda$ ; sodium hydroxide solution, 5 $\lambda$ ; volume of air, 120 $\lambda$ ; size of compensating chamber, 234 $\lambda$ ; lucite cup, 57 $\lambda$ ; sodium hydroxide solution, 5 $\lambda$ ; volume of air, 172 $\lambda$ ; diameter of capillary, 0.22 mm.; cross-section area, 0.037 mm.<sup>2</sup> It is seen that the respiratory rate is a straight line function of time, the deviation of individual readings rarely being as great as 0.01 $\lambda$ .

It appeared that an excellent criterion of reproducibility of results would be to open the respirometer after a reading, reseal it, and take further readings alternating with opening of the instrument. Any systematic error involved in the use of the instrument should be perceived in such a study. This was

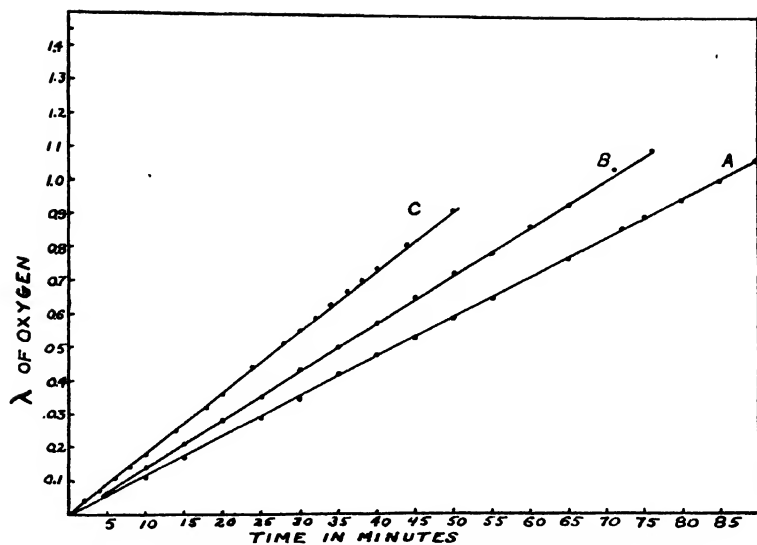


FIG. 2. The oxygen consumption of single *Drosophila* pupae.

TABLE I

*Oxygen Consumption of Drosophila Pupae*

Repeated measurements on the same pupa.

Pupa No.	Observation No.	Respiratory period	Oxygen consumed $\lambda$	Rate $\lambda$ /min.
		min.		
1	1	15	0.195	0.0130
	2	19	0.252	0.0132
	3	15	0.195	0.0130
	4	26	0.334	0.0129
	5	15	0.202	0.0134
	6	25	0.348	0.0138
	7	15	0.202	0.0134
			Mean.....	0.0132 $\pm$ 0.0006
2	1	15	0.248	0.0164
	2	15	0.248	0.0164
	3	15	0.240	0.0160
	4	16	0.266	0.0165
	5	25	0.443	0.0177
	6	15	0.284	0.0185
			Mean.....	0.0169 $\pm$ 0.0016
3	1	35	0.615	0.0177
	2	10	0.175	0.0174
	3	24	0.410	0.0170
			Mean.....	0.0174 $\pm$ 0.0004
4	1	28	0.605	0.0216
	2	20	0.435	0.0218
			Mean.....	0.0217 $\pm$ 0.0001
5	1	76	1.100	0.0145
	2	16	0.252	0.0157
			Mean.....	0.0217 $\pm$ 0.0001
6	1	58	0.860	0.0148
	2	12	0.164	0.0137
			Mean.....	0.0143 $\pm$ 0.0006

accordingly performed using six separate pupae, and making as high as seven determinations on a single organism, each of which was made after opening and resealing the apparatus. The data are given in Table I. Time intervals from 10 to 76 minutes were used to prevent standardization of error. The maximum observed deviation from mean was with pupa 2, which amounted to  $\pm 0.0016\lambda/\text{minute}$ . The mean deviation from mean of all organisms measured was  $0.0004\lambda/\text{minute}$  or an average of 2.4 per cent.

In obtaining the above data, all pupae used were observed later to see that they emerged. Two such organisms which showed a very low respiratory rate failed to emerge and the data were discarded. No effort was made to select pupae of the same size or age in this study.

In comparison with the original respirometer from which this was modified, it is apparent that the accuracy and reproducibility are not diminished. No arrangement for introduction of special gas mixtures is included in the design. It was possible to introduce such mixtures by partially disassembling the apparatus and quickly closing it afterward. It was found definitely easier to operate than the original apparatus, due to its easy assembly. In spite of the lowered metal content of the chamber block and its lack of complete symmetry, it was not affected by temperature change in a manner which should ever cause difficulty in normal circumstances. Its shape permitted simultaneous use of a number of instruments placed side by side, each of them assisting in maintaining constancy of temperature in the others by increasing the mass of metal.

#### BIBLIOGRAPHY

1. Cunningham, B., and Kirk, P. L., *J. Gen. Physiol.*, 1940, **24**, 135.
2. Tyler, A., and Berg, W. E., *Science*, 1941, **94**, 397.

### **CORRECTION**

On page 333, Vol. 25, No. 3, January 20, 1942, the cuts for Figs. 1 and 3 should be interchanged. The legends should remain where they now appear.



# THE ACTIVATION OF PAPAIN TRYPSINASE AS A FUNCTION OF THE NATURE OF THE ACTIVATOR

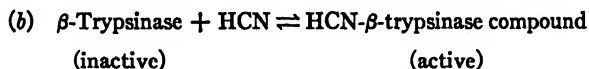
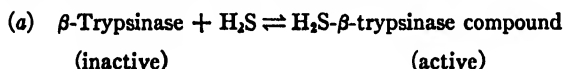
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Papain contains a cysteine-activatable proteinase that hydrolyzes benzoyl-L-arginineamide (1). This enzyme has been shown to have a specificity similar to that of crystalline pancreatic trypsin (2) and therefore has been designated papain trypsinase (3).

Previous experiments have shown that papain trypsinase exists in two inactive forms which can be designated papain- $\alpha$ -trypsinase and papain- $\beta$ -trypsinase (4). Only the  $\beta$ -trypsinase can be activated by HCN. However,  $\alpha$ -trypsinase can be transformed into the  $\beta$ -form by minute amounts of sulfhydryl compounds such as  $H_2S$  or cysteine. The activation of the  $\beta$ -form by an excess of HCN or  $H_2S$  is completely reversed when the activator is removed *in vacuo*. These results have been interpreted to indicate that the reversible activation of papain- $\beta$ -trypsinase consists in the formation of dissociable activator- $\beta$ -trypsinase compounds, as represented below.



It is the purpose of this communication to present additional evidence that the activator-trypsinase compounds obtained with a variety of activators represent different enzymatic entities the proteolytic properties of which depend upon the nature of the activator employed.

*The Simultaneous Action of Two Activators.*—These experiments were performed with a papain preparation obtained from crude papaya latex by  $H_2S$  treatment followed by methanol precipitation and thorough dialysis as described in (4). In all of the experiments to be reported in this paper, the entire activation and hydrolytic procedures were carried out in an atmosphere of nitrogen using the apparatus and technique described in the experimental section.

A sample of the dialyzed papain when activated by treatment with cysteine (0.002 mM per cc.), possessed a proteolytic coefficient<sup>1</sup> towards benzoylarginineamide ( $C_{BAA}$ ) of 0.16 (Table I). The fact that this coefficient represents maxi-

<sup>1</sup> The proteolytic coefficient is defined in (1).



imum activation is indicated by the observation that a tenfold amount of cysteine produced essentially the same coefficient. With  $\text{H}_2\text{S}$  as activator, a coefficient of 0.07 was obtained. In still another experiment, papain was treated simultaneously with cysteine and  $\text{H}_2\text{S}$ , and the ratio of the concentrations of  $\text{H}_2\text{S}$  and of cysteine was 10:1; here  $C_{\text{BAA}}$  was found to be 0.10, a value which lies between the coefficients for cysteine alone and  $\text{H}_2\text{S}$  alone. Subsequently, the  $\text{H}_2\text{S}$  was removed from the activated enzyme solution by evacuation under anaerobic conditions. The remaining solution, which still contained cysteine, but no  $\text{H}_2\text{S}$  gave a  $C_{\text{BAA}}$  of 0.16. This value is identical with that

TABLE I  
*Simultaneous Action of Cysteine and  $\text{H}_2\text{S}$  and of Cysteine and HCN on Papain*  
Substrate, benzoylarginineamide      Temperature, 40°; pH 5.1 to 5.2

Papain concentration in test solution	Activator in test solution	$K_{\text{BAA}}^*$	$C_{\text{BAA}}^*$
mg. P.N. per cc.	mm per cc.		
0.0264	(a) Cysteine (0.002)	0.0039	0.15
0.0132	(b) Cysteine (0.020)	0.0021	0.16
0.0264	(c) $\text{H}_2\text{S}$ (0.020)†	0.0018	0.07
0.0264	(d) Cysteine (0.002) + $\text{H}_2\text{S}$ (0.020)†	0.0026	0.10
0.0264	(e) (d) Evacuated to remove $\text{H}_2\text{S}$	0.0041	0.16
0.0264	(f) (e) + $\text{H}_2\text{S}$ (0.020)†	0.0030	0.11
0.0264	(g) Cysteine (0.005) + $\text{H}_2\text{S}$ (0.020)†	0.0036	0.14
0.0264	(h) (g) Evacuated to remove $\text{H}_2\text{S}$	0.0045	0.17
0.0264	(i) HCN (0.020)†	0.0019	0.07
0.0264	(j) Cysteine (0.00004) + HCN (0.020)†	0.0030	0.11
0.0264	(k) Cysteine (0.00008) + HCN (0.020)†	0.0031	0.12
0.0264	(l) Cysteine (0.002) + HCN (0.020)†	0.0044	0.17

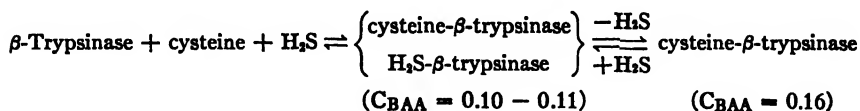
\* For definition of these terms, see (1).

† Incubated for 2 hours at 40° before addition of substrate.

obtained when cysteine alone was employed as activator. Upon addition of  $\text{H}_2\text{S}$  to the evacuated enzyme solution, the proteolytic coefficient for the hydrolysis of benzoylarginineamide dropped to 0.11.

The results, tabulated in Table I, demonstrate a seemingly paradoxical phenomenon in that, under these experimental conditions, the activity of papain  $\beta$ -trypsinase is increased by removing an activator and decreases upon addition of an activator. These results may be explained readily on the basis of the mechanism of activation as represented in the reactions (a) and (b). When papain  $\beta$ -trypsinase is treated with an excess of cysteine, the dissociable compound cysteine- $\beta$ -trypsinase is formed which possesses a  $C_{\text{BAA}}$  of 0.15–0.16. Similarly,  $\text{H}_2\text{S}$  treatment of papain- $\beta$ -trypsinase results in the formation of  $\text{H}_2\text{S}$ - $\beta$ -trypsinase which has a  $C_{\text{BAA}}$  of 0.07. When both activators are present,

a portion of the trypsinase will combine with  $\text{H}_2\text{S}$  and the remainder will combine with cysteine. The concentrations of  $\text{H}_2\text{S}$ - $\beta$ -trypsinase and of cysteine- $\beta$ -trypsinase will depend upon their dissociation constants and upon the relative amounts of  $\beta$ -trypsinase,  $\text{H}_2\text{S}$ , and cysteine present. Thus, when the  $\text{H}_2\text{S}$  concentration is ten times that of cysteine, it should be expected that  $\text{H}_2\text{S}$ - $\beta$ -trypsinase should be formed at the expense of cysteine- $\beta$ -trypsinase and that the coefficient for the hydrolysis of benzoylarginineamide should be lower than the maximum value for the cysteine- $\beta$ -trypsinase and higher than the maximum value for the  $\text{H}_2\text{S}$ - $\beta$ -trypsinase.



Removal of  $\text{H}_2\text{S}$  from the mixture of the two enzymes completely dissociates the  $\text{H}_2\text{S}$ - $\beta$ -trypsinase, leaving cysteine- $\beta$ -trypsinase as the only enzyme. However, subsequent addition of sufficient  $\text{H}_2\text{S}$  to reestablish the 10:1 ratio of  $\text{H}_2\text{S}$ :cysteine, brings about partial dissociation of the cysteine- $\beta$ -trypsinase and re-synthesis of the  $\text{H}_2\text{S}$ - $\beta$ -trypsinase.

It would be predicted also, that a smaller difference in the concentrations of  $\text{H}_2\text{S}$  and cysteine, when used together to activate the  $\beta$ -trypsinase, would result in the formation of a mixture of enzymes whose coefficient would be nearer that of cysteine- $\beta$ -trypsinase. This is borne out by data included in Table I where it is seen that the mixture of active compounds formed by activating  $\beta$ -trypsinase with  $\text{H}_2\text{S}$  and cysteine in the ratio 4:1, possesses a coefficient of 0.14. As before, removal of the  $\text{H}_2\text{S}$  *in vacuo* results in an increase in activity to a value closely approximating that found for cysteine  $\beta$ -trypsinase.

Furthermore, it may be concluded from the results of Table I that both  $\text{H}_2\text{S}$  and cysteine activate the same enzymatic component of papain. If  $\text{H}_2\text{S}$  were activating one component of papain and cysteine another to give two active  $\beta$ -trypsinases both capable of hydrolyzing benzoylarginineamide, then the simultaneous use of both activators in excess should produce a proteolytic coefficient equivalent to the sum of the maximum coefficients obtained with  $\text{H}_2\text{S}$  alone and cysteine alone. This is not found to be the case.

Similarly, it may be concluded that  $\text{HCN}$  and cysteine activate the same enzymatic component of papain. It will be recalled that only the  $\beta$ -trypsinase is activated by  $\text{HCN}$ . The low  $C_{\text{BAA}}$  value obtained after activation of the papain preparation with  $\text{HCN}$  (Table I) indicates that much of the trypsinase is in the inactivatable  $\alpha$ -form. However, a cysteine concentration of  $4 \times 10^{-5}$  mm per cc. of test solution is sufficient to bring about complete transformation of  $\alpha$ -trypsinase to the  $\beta$ -form, thus making it possible to obtain the maximum proteolytic coefficient for  $\text{HCN}$ - $\beta$ -trypsinase ( $C_{\text{BAA}} = 0.11\text{--}0.12$ ). It is apparent that when cysteine and  $\text{HCN}$  are used simultaneously and in excess to

activate the trypsinase, the  $C_{BAA}$  value (0.17) obtained is significantly less than the sum of the  $C_{BAA}$  values (0.26) for the cysteine- $\beta$ -trypsinase and HCN- $\beta$ -trypsinase.

*The Proteolytic Coefficient As a Function of the Nature of the Activator.*—The experiments described in this section were performed with a papain fraction ("Papain B" of (1)) obtained by a procedure similar to that described by Balls and Lineweaver for their crystalline papain (5). Since this papain fraction was found to hydrolyze benzoylarginineamide in the presence of either  $H_2S$  or cysteine considerably faster than the papain used in the experiments of Table I, it may be regarded as a more concentrated preparation of papain trypsinase. Consequently, this preparation was chosen for the comparison of the  $C_{BAA}$  values in the presence of four different activators, namely:  $H_2S$ , cysteine, glutathione, and HCN.

The enzyme preparation was thoroughly dialyzed against distilled water before use. In the highest concentrations applied in these experiments, the dialyzed enzyme preparation contained insufficient natural activator to cause any appreciable hydrolysis of benzoylarginineamide ( $C_{BAA} = 0.00$ ). Furthermore, the preparation contained most of the trypsinase in the  $\alpha$ -form as is apparent from the relatively low proteolytic coefficient obtained by the use of HCN as the activator ( $C_{BAA} = 0.07$ ). For the determination of the maximum proteolytic coefficients with the sulfhydryl activators, the presence of  $\alpha$ -trypsinase was of no consequence since traces of each of these activators are capable of transforming  $\alpha$ -trypsinase to the  $\beta$ -form. In the determination of the maximum proteolytic coefficient with HCN, sufficient cysteine ( $4 \times 10^{-5}$  mm per cc.) was employed to ensure complete transformation of  $\alpha$ -trypsinase to the  $\beta$ -form.

The comparative activities of the activator-trypsinase compounds are presented in Table II. Each of the activators was shown to be present in sufficient excess to bring about maximum activity, since in each case doubling the activator concentration failed to increase the rate of hydrolysis. For each of the activators the velocity constants obtained were found to be directly proportional to the enzyme concentration.

The results show that the proteolytic coefficients obtained for papain trypsinase are dependent upon the nature of the activator employed. Under the conditions of maximum activity the HCN-activated trypsinase hydrolyzes the substrate at more than twice, and the cysteine- or glutathione-activated trypsinase at more than three times the rate found for the  $H_2S$ -activated trypsinase. In effect, therefore, the compounds of trypsinase with different activators behave as different enzymatic entities.

Cysteine-papain trypsinase also hydrolyzes benzoyl-*L*-lysineamide (2). Experiments are in progress to determine whether the ratio  $C_{BAA}/C_{BLA}$  for the various activator- $\beta$ -trypsinase compounds is the same; *i.e.*, whether these compounds may be regarded as homospecific enzymes (3).

TABLE II

*Action of Several Activators on Papain*

Substrate, benzoylarginineamide

Temperature, 40°; pH 5.0 to 5.3

Activator	Papain Concentration in test solution	Activator concentration mm per cc. of test solution				Average C <sub>BAA</sub>	
		0.020		0.040			
		K <sub>BAA</sub>	C <sub>BAA</sub>	K <sub>BAA</sub>	C <sub>BAA</sub>		
H <sub>2</sub> S*	mg. P.N. per cc. 0.0105	0.0013	0.12	—	—	0.12	
		0.0012		—	—		
		0.0014		—	—		
	0.0158	0.0019	0.13	—	—		
		0.0023		—	—		
		0.0020		—	—		
		0.0023		—	—		
	0.0210	0.0025	0.12	0.0023	0.12		
		0.0024		0.0023			
0.0027		0.0027					
Cysteine	0.0063	0.0022	0.37	—	—	0.38	
		0.0024		—	—		
		0.0024		—	—		
	0.0085	0.0035	0.41	—	—		
		0.0035		—	—		
		0.0035		—	—		
	0.0105	0.0037	0.38	0.0039	0.37		
		0.0041		0.0038			
		0.0042		0.0041			
Glutathione	0.0063	0.0026	0.38	—	—	0.37	
		0.0023		—	—		
		0.0024		—	—		
	0.0105	0.0038	0.38	0.0040	0.36		
		0.0039		0.0036			
		0.0043		0.0039			
HCN*‡	0.0053	0.0017	0.32	—	—	0.30	
		0.0016		—	—		
		0.0018		—	—		
	0.0084	—	—	0.0023	0.27		
		—		0.0023			
	0.0105	0.0028	0.30	—	—		
		0.0031		—	—		
		0.0033		—	—		

\* Incubated for 2 hours at 40° before addition of substrate.

† In this series the test solution also contained  $4 \times 10^{-5}$  mM cysteine per cc.

## EXPERIMENTAL

*Apparatus.*—The apparatus used for carrying out enzymatic experiments in nitrogen is shown diagrammatically in Fig. 1. The apparatus is constructed of Pyrex glass and standard interchangeable ground glass joints and stopcocks.

A description of the procedure used in the experiments on cysteine activation (Table II) will be given to illustrate the technique.

A slow stream of nitrogen, purified by passing the gas over hot reduced copper, enters the apparatus as shown at the left of the diagram. The glass tubing to vessels

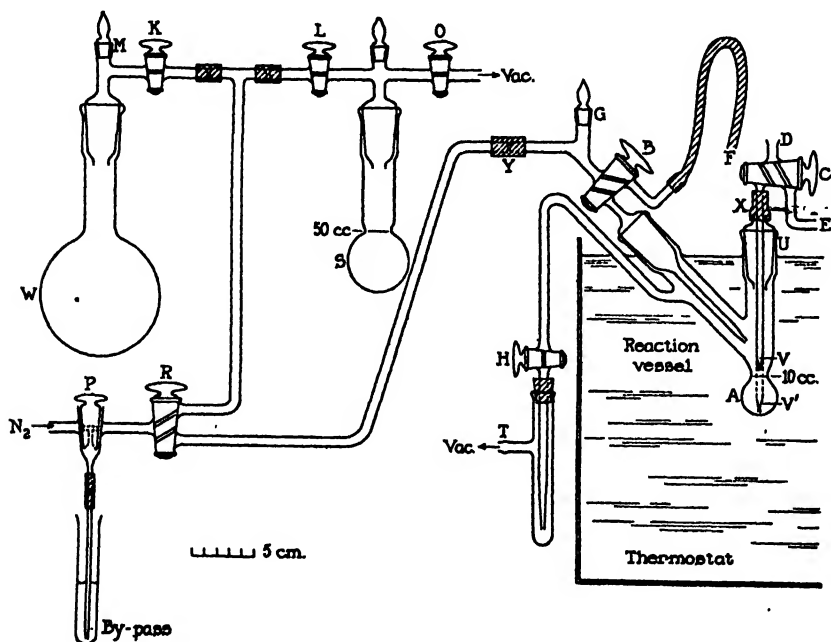


FIG. 1. Apparatus for anaerobic enzymatic experiments.

*W* and *S* is flushed out with nitrogen and the stopcocks *K* and *L* are closed. Flask *W* (500 cc.), is filled with distilled water and the water is boiled for several minutes with *M* open to remove air. The stopper is replaced in *M* and the vessel is allowed to cool to room temperature. Nitrogen is admitted through *K* until atmospheric pressure is established. In this, and in subsequent operations, the stopcock *P* and the by-pass may be used to indicate when pressure equilibrium has been established. When nitrogen is not being used, it is allowed to escape through the by-pass.

Air-free water is obtained from *W* as needed by opening *K* to provide a positive nitrogen pressure in *W* so that air cannot enter *W* when *M* is opened; inserting a pipette equipped with a tight rubber collar into the water through *M*; allowing nitrogen pressure to fill the pipette to the desired volume; removing the pipette and replacing the stopper in *M*; closing *K*.

The substrate (benzoylarginineamide·HCl·H<sub>2</sub>O 2.074 gm.) is weighed into the vessel *S* (50 cc.) and the flask is half filled with air-free water. By successively evacuating the vessel through *O* and refilling with nitrogen through *L* several times, air contained in the vessel is removed. The solution is then diluted to 50 cc. with air-free water. Air-free substrate solution (0.125 M) can be removed as needed by following the procedure described above for obtaining air-free water.

The reaction vessel is connected at *Y* by means of a short length of pressure tubing and nitrogen is passed through *G* for several minutes to remove air from the system from *R* to *G*. The stopper is replaced in *G*, *F* is connected to *D*, and nitrogen is

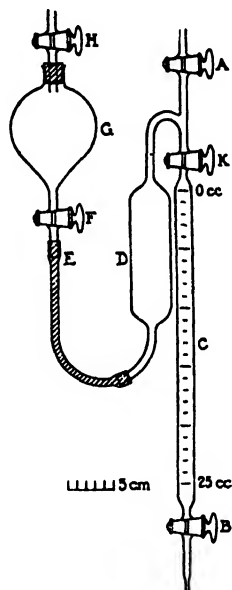


FIG. 2. Apparatus for storage and delivery of air-free H<sub>2</sub>S solutions of constant titre.

passed through *B-F-D-E* while the materials under test are introduced into *A*. The entire assembly included in the ground joint at *U* is lifted and the desired volume of enzyme solution (usually 1.0 or 2.0 cc.) and 1.0 cc. of 0.2 molar citrate buffer (pH 5.0) are pipetted into *A*. The assembly is replaced in *U* and the system is evacuated (cautiously at first to minimize foaming) by means of a water pump at *T*. The stopcocks at *H* and *C* are closed and nitrogen is admitted through *B* until pressure equilibrium is attained. Evacuation and refilling with nitrogen is repeated several times to remove air from the vessel and contents. The required volume of air-free 0.1 M cysteine solution (2.0 cc. for a cysteine concentration of 0.020 mM per cc. of test solution) is now added through *G* by means of a rubber-collared pipette. The solution is forced into the reaction vessel by a stream of nitrogen passing from *B* to *H*. Substrate solution (4.0 cc. of 0.125 M solution to give a concentration of 0.05 mM/cc. of test solution) is then added in exactly the same manner. The reagents are

rinsed into the vessel and the mixture is diluted to 10.0 cc. by adding air-free water through *G*.

The end of the sampling tube *V* is lowered to position *V'* by sliding the tube through the rubber sleeve *X*. The solution in *A* is mixed by shaking or by bubbling nitrogen through *V'* for several seconds. *F* is disconnected from *D*. Samples are removed for titration (6) by forcing the solution under nitrogen pressure from *B*, into the well at *D* (volume of well approximately 0.3 cc.) from which 0.2 cc. samples can be taken readily by means of a micro pipette. The excess of solution in *D* is discarded through *E*. *F* is again connected to *D* and, after nitrogen has flushed all air from *D*, the solution in the sampling tube is returned to *A* by nitrogen pressure at *D* with *H* open. Subsequent samplings at the desired time intervals are accomplished similarly. Between samplings, stopcocks at *C*, *B*, and *H* are kept closed and the nitrogen escapes through the by-pass.

With practice, 1 minute is required to remove 2 titration aliquots and to return the solution in the sampling tube to the reaction vessel. At the start of an experiment 3 to 4 minutes are required to add substrate solution, dilute to volume, mix, and remove the initial samples. In successive sampling, the tube *V* and the well *D* are rinsed free of the previous sample by discarding several portions of the test solution through *E* before the titration sample is pipetted.

Experiments of the type described in Table I can be carried out conveniently in the apparatus by employing essentially the same technique described above. In the experiments requiring the *in vacuo* removal of  $\text{H}_2\text{S}$ , a trap containing lead acetate solution is placed between *T* and the pump. Complete removal of  $\text{H}_2\text{S}$  is effected by repeated evacuation and refilling with nitrogen.

**Activator Solutions.**—With the exception of  $\text{H}_2\text{S}$ , activator solutions were prepared freshly immediately before use. Air-free 0.1 M cysteine, glutathione, and HCN solutions were prepared by placing the proper amount of cysteine hydrochloride, glutathione, or KCN in an air-free volumetric flask and diluting to volume with air-free water and sufficient N NaOH or N HCl to make the final solution pH 5.0. To prevent volatilization of HCN, this activator solution was prepared and kept in an ice bath.

Air-free  $\text{H}_2\text{S}$  was prepared and stored in the apparatus shown in Fig. 2.

The bulb *G* is detached at *E* and the rest of the apparatus is filled with  $\text{H}_2\text{S}$  by passing the gas through the opening at *E* for several minutes. *G* is filled with air-free water and the water is saturated with  $\text{H}_2\text{S}$  at  $0^\circ\text{C}$ . The bulb *G* is connected to *E* and the apparatus is inverted, so that the  $\text{H}_2\text{S}$  solution fills *D* and *C*. *B* and *K* are closed and the apparatus is placed upright. With *H* and *F* open,  $\text{H}_2\text{S}$  pressure is applied at *A* to force the  $\text{H}_2\text{S}$  solution above *K* to the bottom of *D*. All stopcocks are closed and the entire set-up is kept in a constant temperature cold room.

To remove a sample of air-free  $\text{H}_2\text{S}$  solution, stopcock *K* is opened and a measured amount is drawn off at *B*. If the pressure in *D* becomes too low to permit delivery from *B*, raising the bulb *G* with *F* and *H* open will reestablish the necessary pressure.

Prepared and stored in the manner described, the  $\text{H}_2\text{S}$  solution is perfectly clear and its concentration remains constant for long periods. The apparatus provides a readily available source of known amounts of  $\text{H}_2\text{S}$ . Standardization of the solution is accomplished by iodometric titration (7). A solution of  $\text{H}_2\text{S}$  prepared and stored

in this manner was 0.18 M when prepared and had exactly the same molarity 3 months later.

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## PURIFICATION OF THROMBIN

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The coagulation of blood is customarily considered to proceed in two stages. During the first stage a series of reactions results in the activation of prothrombin to thrombin. In the second stage thrombin brings about the conversion of fibrinogen to fibrin, which composes the solid framework of the blood clot. The mechanism of these reactions has yet to be unraveled, despite a great deal of work with crude materials. Consequently, recent years have seen increasing attention devoted to the purification of the central factors in the clotting system (1-7).

The purpose of this paper is to present a new method for the purification of thrombin, which regularly yields a product the specific activity of which is 100-175 times the potential specific activity of whole plasma.

### *Preparation of Prothrombin*

The first two steps of this method were derived from the procedure of Mellanby (1). The present modification has yielded consistently good results when employed on fresh plasma from carefully collected blood.

Oxalated beef plasma which was no more than 4 days old was fractionated as outlined in Table I. The euglobulins were precipitated from diluted plasma at pH 5.2. From the precipitate, which contained prothrombin, thrombokinase, and fibrinogen, most of the prothrombin was extracted by dilute calcium bicarbonate. During the earlier work with this procedure, it occasionally happened that the finely suspended globulin would flocculate heavily upon the addition of the calcium bicarbonate, and thus render the extraction inefficient. It appeared that the success of the operation depended on extracting the prothrombin before the fibrinogen had progressed too far toward coagulation. Therefore the time limits recorded in Table I were closely observed. Only scrupulously clean apparatus was used, since extraneous matter often accelerates coagulation.

The prothrombin was precipitated from the extract between 0.28 and 0.55 saturated ammonium sulfate. It was then made up to approximately 1 mg. protein nitrogen per ml. and twice reprecipitated by 0.45 saturated ammonium sulfate. The final precipitate, dissolved in a small quantity of distilled water, gave an amber, opalescent solution, the pH of which was 5.4. After dialysis

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TABLE I  
*Preparation of Prothrombin and Conversion to Thrombin*

	No.	Units ml. plasma	mg. P.N. ml. plasma	Units mg. P.N.
Oxalated beef plasma.....		1,900	11	170
In 4 aluminum vats: 3.5 liters of 1 per cent acetic acid stirred with 201.4 liters distilled water. 10.6 liters plasma added, stirred gently. Allowed to settle 2 hrs. at 20°C. Supernatant siphoned off. Euglobulin precipitate.....	1	1,500	1.6	940
No. 1 suspended in remaining mother liquor and packed by centrifugation. Cake ground in mortar and suspended in 20 liters distilled water at 20°C. Suspension distributed among seven 6 liter Erlenmeyer flasks. 3 liters dilute calcium bicarbonate added to each flask which was immediately closed by a rubber stopper and mixed by inversion. Solid kept in suspension by occasional inversion of flask. After 20 min. at 20°C. flasks kept in water baths at 6°C. for 75 min. Supernatant decanted through coarse folded filters at 6°C. Sediment packed by centrifuge in rubber stoppered tubes. Supernatant poured into filters.				
Combined filtrates = extract.....	2	<u>960</u>	0.17	5,600
39 liters No. 2 stirred by motor while 13.6 kg. solid ammonium sulfate added slowly. Stirred 2.5 hrs. at 20°C. Plus 80 gm. Filter-cel. Filtered with suction. Filter cake stirred 15 min. with 1 liter 0.9 per cent sodium chloride and filtered with suction.				
Filter cake.....	3			
Filtrate and washings.....	4			
No. 3 stirred 15 min. with 350 ml. 0.9 per cent sodium chloride. Filtered with suction. Filtrate and washings.....	5			
No. 4 and No. 5 combined, 1,550 ml. Plus 600 ml. saturated ammonium sulfate to approximately 0.28 saturated. Centrifuged. Precipitate discarded. Supernatant plus 600 ml. saturated ammonium sulfate to approximately 0.45 saturated. Supernatant discarded. Precipitate.....	6	<u>720</u>	0.12	6,000
No. 6 dissolved in distilled water to 1 liter of solution. Plus 818 ml. saturated ammonium sulfate, centrifuged. Precipitate.....	7			
No. 7 dissolved in distilled water to 1 liter of solution. Plus 818 ml. saturated ammonium sulfate. Centrifuged. Precipitate.....	8			
No. 8 dissolved in distilled water to 90 ml. solution. Concentrated prothrombin.....	9	<u>640</u>	0.11	5,800
No. 9 dialyzed against 3.5 liters 0.09 saturated ammonium sulfate at 6°C. 1 week at 20°C. then stored at 1°C. Crude thrombin.....	10	<u>260</u>	<u>0.11</u>	2,400

The underlined figures are average values from six preparations.

the concentrated prothrombin was set aside to change to thrombin. The preparation up to the point of dialysis was completed within 24 hours.

### *"Spontaneous" Activation of Concentrated Prothrombin*

The conversion of prothrombin to thrombin under these conditions proved to be particularly interesting, especially when compared to the behavior of the extract from which it had been concentrated. The pH of the extract was usually 6.8. When stored in the ice box it lost about 10 per cent of its prothrombin per week, but produced no detectable thrombin. Even when calcium and physiologic saline were added, it did not develop thrombin unless an activator such as cephalin or lung extract were included.

In contrast, the concentrated prothrombin changed to thrombin without any added activator. Furthermore, ionized calcium was unnecessary. Quantitative determinations on the concentrate showed the calcium to be 0.002 M; and this was probably bound to protein. The essential condition for "spontaneous" activation seemed to be the concentration of the biologic material. Indeed, activation could be retarded merely by dilution of the concentrate. The data on hand do not permit a decision as to whether it was the prothrombin itself or some accompanying factor which was responsible for this phenomenon.

In a series of experiments performed at pH 7.4 it was found that 0.026 M oxalate failed to retard activation. Although the conversion was accelerated by crystalline trypsin (*cf.* 8) it was unaffected by crystalline trypsin inhibitor. Conditions which favored rapid activation did not necessarily lead to better yields of thrombin. For example, activation was much faster at pH 6.5 than at pH 5.4 but thrombin was destroyed more rapidly at the higher pH, resulting in a lower final yield. Conditions for optimal yield were found to be 0.1 saturated ammonium sulfate at pH 5.4 and the temperature variations mentioned in Table I.

### *Fractionation of Crude Thrombin*

Preliminary experiments had shown that thrombin was soluble in 0.45 saturated ammonium sulfate, whereas crude prothrombin was not. In the fractionation of prothrombin (Table I) particular care had been taken to eliminate protein which was soluble in 0.45 saturated ammonium sulfate. Therefore, proteins insoluble in that solvent constituted almost all the impurities which were present in the concentrated prothrombin, and consequently, in the crude thrombin. On the other hand, a large proportion of the thrombin itself would be expected to remain dissolved in 0.45 saturated ammonium sulfate when the crude thrombin was fractionated. Such was the case, as is shown in Table II, where the most soluble fraction, 9/15 III, contained almost half the total activity, but only 3 per cent of the total protein. When 9/15 III was again treated to remove protein soluble in 0.45 saturated ammonium sulfate, the specific activity rose from 15,100 to 22,000. From there on fractionation was difficult but clearly showed that the protein was not yet homogeneous. As may be seen from the data in Table II, the thrombin was

TABLE II  
*Fractionation of Thrombin*

	No.	Per cent saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Total mg. P.N.	Units mg. P.N.
Crude thrombin from 3 preparations plus distilled water to 3 liters plus 2,450 ml. saturated ammonium sulfate and 75 gm. Filter-cel. Filtered.				
Filtrate and washing.....	1	0.45		
Cake stirred in 3,300 ml. distilled water. Plus 2,700 ml. saturated ammonium sulfate. Filtered.				
Filtrate and washing.....	2	0.45		
Residue.....	9/15/ I		4,420	500
No. 1 and No. 2 combined. Vol. 11.1 liters. Plus 2.3 kg. ammonium sulfate and 24 gm. Filter-cel. Filtered.				
Filtrate discarded.....		0.75		
Cake extracted twice with 900 ml. 0.45 saturated ammonium sulfate. Residue.....	9/15 II		52	5,300
Combined extracts plus 1,030 ml. saturated ammonium sulfate. Centrifuged. Supernatant discarded.....		0.65		
Precipitate.....	9/15/ III		122	15,100
9/15 III dissolved in 35 ml. distilled water. Vol. 48 ml. Plus 24 ml. saturated ammonium sulfate. Centrifuged.				
Supernatant.....	3	0.45		
Residue extracted twice with 150 ml. 0.45 saturated ammonium sulfate. Combined extracts.....	4	0.45		
Residue.....	10/2 I		27	2,900
No. 3 and No. 4 combined. Vol. 370 ml. Plus 212 ml. saturated ammonium sulfate. Centrifuged.				
Supernatant.....		0.65		
Precipitate.....	10/2 II		69	22,000
10/2 II dissolved in 70 ml. distilled water. Vol. 77 ml. Plus 30 ml. saturated ammonium sulfate plus 130 ml. (0.99 saturated ammonium sulfate, 0.04 M pH 5.2 acetate). Centrifuged. Supernatant discarded.....		0.70		
Precipitate extracted 3 times with 180 ml. (0.5 saturated ammonium sulfate, 0.08 M pH 5.2 acetate). Residue.....	10/20 I		20	11,100
Combined extracts plus 366 ml. (0.99 saturated ammonium sulfate, 0.04 M pH 5.2 acetate). Centrifuged.				
Supernatant discarded.....		0.70		
Precipitate.....	10/20 II		57	20,000
10/20 II dissolved in 70 ml. (0.35 saturated ammonium sulfate, 0.08 M pH 5.2 acetate). Vol. 76 ml. ....		0.38		
Plus 16.5 ml. (0.98 saturated ammonium sulfate, 0.08 M pH 5.2 acetate). Centrifuged. Precipitate.....	11/10 A	0.49	13.5	15,500
Supernatant plus 2 ml. 0.98 SAS.* Centrifuged. Precipitate.....	11/10 B		6.3	25,600

TABLE II—*Concluded*

	No.	Per cent saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Total mg. P.N.	Units mg. P.N.
Supernatant plus 1 ml. 0.98 SAS. Centrifuged.				
Precipitate.....	11/10 C		3.7	30,500
Supernatant plus 1 ml. 0.98 SAS. Centrifuged.				
Precipitate.....	11/11 A		3.4	29,700
Supernatant plus 30.5 ml. 0.98 SAS. Centrifuged.				
Supernatant discarded.....		0.62	2.2	
Precipitate extracted 10 times with 10 ml. portions of (0.55 saturated ammonium sulfate, 0.08 M pH 5.2 acetate).				
Total for 10 extracts.....	E1-E10	0.55	8.1	12,300
Residue extracted with 10 ml. portions of (0.53 saturated ammonium sulfate, 0.08 M pH 5.2 acetate).				
Total for extracts.....	E11-E15	0.53	4.3	16,800
	E16-E17		1.4	26,100
	E18-E22		2.5	31,000
	E23-E25		0.9	
Residue.....			4.0	22,000

\* 0.98 SAS (0.98 saturated ammonium sulfate, 0.08 M pH 5.2 acetate).

accompanied by at least two other proteins, one of which was slightly less soluble (in 11/10 A and 11/10 B) and the other slightly more soluble (in E1-E17) than thrombin.

It should be mentioned that the fractionation of Table II was the best of several. In other fractionations the specific activity stopped between 17,000 and 30,000. In one case a sample was treated by several successive additions of saturated ammonium sulfate and gave twelve successive precipitates with a specific activity about 17,000. The reason for this may be surmised from a consideration of the closely similar solubilities of thrombin and the final impurities. The proportion of these impurities in the crude thrombin might determine at what point fractionation would become impracticable. It must be left to future work to determine to what extent the final impurities might be derived from thrombin itself, and whether there are varieties of thrombin molecules with different specific activities (*cf.* 9).

#### *Some Properties of the Thrombin Preparations*

From the data in Table II it can be calculated that 70 per cent of the activity present in sample 9/15 III could be accounted for in the 32 fractions derived from it in the course of the next 2 months. In another experiment, on a sample having a specific activity of 13,000, it was found that thrombin was most stable at pH 5.2. Solutions containing 1 mg. protein nitrogen per ml. of 0.35

saturated ammonium sulfate at pH 5.2 lost less than 10 per cent of their activity in 4 days at room temperature.

Solutions having 1 mg. protein nitrogen per ml. and a specific activity of 20,000 or more, were water-clear and had an almost imperceptible yellow tinge. Dr. Alexandre Rothen kindly performed electrophoretic tests on several samples. On Fig. 1 is shown the best pattern obtained, from a sample having 29,000 thrombin units per mg. protein nitrogen. Solubility curves on several samples were of the rounded, solid solution type indicating inhomogeneity (10).

A few tests for proteolytic action on casein (pH 7.6) were performed with a thrombin sample having a specific activity of 9,000. In the standard test (11) 0.1 mg. protein nitrogen caused no change in the viscosity of casein in 1 hour. The effect of thrombin on the formol titration of casein was about 1/10,000 that of crystalline trypsin. Of course, these tests do not exclude the possi-

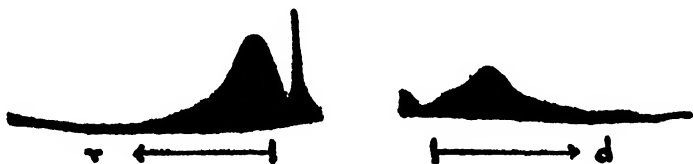


FIG. 1. Electrophoretic pattern of a thrombin preparation having a specific activity of 29,000. The solvent was (0.02 M acetate buffer pH 5.2; 0.08 M sodium chloride). Thrombin migrated toward the anode. The photograph was taken 2 hours, 47 minutes from start. The slender spike is a buffer effect and does not represent another protein component.

bility that thrombin has some proteolytic action of fibrinogen. As noted also by Seegers (5) purified thrombin has no fibrinolytic activity.

#### DISCUSSION

If it be assumed that 1 molecule of prothrombin is converted to 1 molecule of thrombin having approximately the same molecular weight it follows that the same number would designate the specific activity of pure thrombin and the potential specific activity of pure prothrombin. From the results herein presented, that number would be above 30,000. 2,000 prothrombin units per ml. plasma would then represent less than 0.067 mg. prothrombin nitrogen. Since there are about 11 mg. protein nitrogen per ml. plasma, prothrombin comprises less than 0.6 per cent of the plasma proteins.

By the present assay method normal beef plasma contains 2,000 prothrombin units per ml. and human plasma, 1,700 units per ml. By the Iowa method of assay, there are 314 Iowa units of prothrombin per ml. beef plasma, and 325 Iowa units per ml. human plasma (3, 5). Thus, the present unit is 1/6 the

Iowa unit. Using this factor to convert the assay values of Seegers to the present unitage it can be calculated that the usual thrombin preparation obtained by Seegers had 28,000 units per mg. protein nitrogen, and that his best preparation had a specific activity of 43,000. The highest single assay value obtained during the present work was 36,000. Further comparison of the two methods might prove fruitful since their strong features are quite different.

### *Materials and Methods*

*Buffer for Fibrinogen.*—This was a 0.9 per cent solution of sodium chloride containing 0.0067 M phosphate buffer pH 7.4 and 0.013 M potassium oxalate.

*Buffer for Assays.*—50 ml. 0.1 M sodium diethylbarbiturate were mixed with 36 ml. 0.1 M hydrochloric acid and diluted to 250 ml. with oxalated saline. The final concentration of oxalate was 0.0008 M.

*Calcium Chloride.*—0.11 M.

*Calcium Bicarbonate.*—Distilled water was shaken with excess CaO. The filtered solution was diluted tenfold with distilled water. CO<sub>2</sub> was rapidly bubbled through the 0.1 saturated Ca(OH)<sub>2</sub> until the solution was yellow to brom thymol blue. Air was then bubbled through until the solution was orange to phenol red. The bottle was closed tightly with a rubber stopper.

*Beef Plasma.*—Beef blood was obtained at the slaughter house. The blood gushing from a severed vessel was caught directly into 150 ml. of 10 per cent potassium oxalate in a wide mouthed 4 liter jar. The mouth of the jar was not allowed to touch the wounded tissue and any blood which flowed over the wound was rejected. Within a few seconds the jar was  $\frac{3}{4}$  full and it was quickly stoppered and shaken. After the blood had cooled in the ice box the plasma was collected by centrifugation and stored at 6°C.

*Fibrinogen.*—17 ml. of saturated ammonium sulfate were slowly added to 50 ml. of beef plasma. 15 minutes later the mixture was centrifuged lightly for 5 minutes and the precipitate was immediately stirred thoroughly with 200 ml. 0.25 saturated ammonium sulfate. After 15 minutes the residue was collected by centrifugation and dissolved in 75 ml. of buffer. Kept in the ice box these solutions were fairly stable and as will be seen below were sufficiently uniform for assay purposes. The small portions which were warmed to room temperature for each assay were not returned to the cold stock solution since long exposure to room temperature affects the clotting response.

*Lung Extract.*—0.5 gm. dried beef lung was extracted by grinding with 10 ml. of 0.9 per cent sodium chloride.

*Assay of Thrombin Activity.*—All reagents were at room temperature. The thrombin solution was diluted with buffer and to 1.0 ml. of the diluted thrombin was added 0.2 ml. calcium chloride. Then 0.1 ml. of this mixture was blown directly into 0.3 ml. of fibrinogen in a 10 × 75 mm. pyrex tube. The contents were immediately mixed by rapid oscillation. The clotting time was taken as the interval between the addition of the thrombin and the instant when the clot could hold its position with the



tube inverted. It was determined empirically that the clotting time was inversely proportional to the concentration of thrombin under these conditions. A unit was defined as the amount which gave a clot in 300 seconds. The dilutions were usually arranged so that 3 to 10 units were estimated. Example: A solution of thrombin was diluted 1/16, then 3/10, and the calcium chloride added. 0.1 ml. of this produced a clot in 50 seconds. The original solution contained  $16 \times \frac{10}{3} \times \frac{12}{10} \times 10 \times \frac{300}{50} = 3340$  thrombin units per ml.

A standard solution was prepared by precipitating thrombin with ammonium sulfate and dissolving the precipitate in glycerin. Kept in the ice box this solution maintained its titer for 8 months. During this time the titers obtained with eight fresh preparations of fibrinogen varied from 11,700 to 13,500 units per ml. Only one fresh fibrinogen preparation (from anemic blood) had to be discarded because it gave values outside this range. When a fibrinogen solution had aged to the extent that it gave low titers for the standard thrombin it was replaced by a fresh preparation.

*Prothrombin Assay.*—0.9 ml. of a buffer dilution of prothrombin was mixed with 0.1 ml. lung extract and 0.2 ml. calcium chloride. At intervals the activation mixture was assayed for thrombin, the plateau value being used to calculate the potential activity of the prothrombin.

*Protein Nitrogen.*—Turbidity method (9). The turbidity curve was standardized by Kjeldahl determination on a sample having a specific activity of 18,000.

*Tests for Proteolysis.*—Northrop and Kunitz (11).

#### SUMMARY

1. Under certain conditions crude prothrombin changes to thrombin without the addition of extraneous activators and in the absence of ionic calcium.
2. Thrombin is soluble in 0.45 saturated ammonium sulfate, whereas crude prothrombin is not.
3. Partially purified thrombin is comparatively stable in concentrated ammonium sulfate solutions at pH 5.2.
4. A method based on the above facts yields a thrombin preparation the specific activity of which is 100 to 175 times the potential specific activity of whole plasma.

This work was carried out under the guidance of Dr. John H. Northrop. The author is also indebted to Mr. J. F. Gettemans who helped work up 400 liters of blood.

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# THE METABOLISM OF TISSUE CULTURES\*

## I. PRELIMINARY STUDIES ON CHICK EMBRYO

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The study of tissue metabolism by classical methods has certain serious limitations which are obviated by the use of tissue cultures. In investigating the mechanism of growth it is particularly desirable to use intact cells, whose growth may be controlled during the period of observation. The present study deals with metabolic processes in cultures of embryonic tissue grown under normal conditions.

All experiments were conducted on chick embryo tissue, usually 9 to 13 days old. Muscle, heart, and liver tissue were explanted together and grown by the roller bottle technique described by Shaw, Kingsland, and Brues (1). Unless otherwise stated, cultures were grown in an artificial medium similar to that described by Baker (2), but without added serum.<sup>1</sup> In any one experiment all culture bottles contained the same amount of medium per unit weight of wet tissue; a typical culture thus had 160 mg. tissue and 4 cc. of medium. Changes in composition of the medium resulting from metabolism of the explanted tissue were then followed by analytical methods.

### Glucose Utilization

Previous observations have shown that tissues can use an amount of glucose greater than their own dry weight in 48 hours (Krontowski (3)), and that the

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<sup>1</sup> 100 cc. of the modified Baker's solution contained:

	mg.
NaCl.....	720
KCl.....	18
CaCl <sub>2</sub> ·2H <sub>2</sub> O.....	23.8
MgCl <sub>2</sub> ·6H <sub>2</sub> O.....	9
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O.....	5.2
NaHCO <sub>3</sub> .....	100
Witte's peptone.....	675
Glucose.....	100
Cysteine HCl.....	9
Glutathione.....	1
Ascorbic acid.....	0.25
Thiamin HCl.....	0.5

amount used decreases with the age of the tissue (Lebensohn (4)). The effect of glucose concentrations higher than those found in physiological media has received considerable attention. Demuth (5) stated that growth in cultures increased with increasing glucose concentration. Ebeling (6) observed increased growth in cultures of fibroblasts whose glucose was raised from 200 to 1150 mg. per cent; at higher levels glucose appeared to inhibit growth and to become toxic. Willmer (7), using saline mixture media, found the total amount of cell migration to be directly proportional to glucose concentration up to 1000 mg. per cent, although the tissues were more active in concentrations of 200 mg. per cent as long as enough glucose was present.

TABLE I  
*Glucose Utilization in Relation to Initial Concentration*

Concentration expressed as mg. per cent in media.

Rate = mg. glucose per 100 mg. tissue per day.

Initial concentration . . . . .	622		407		198		86	
Rate on 1st day . . . . .		2.6		2.7		2.0		1.4
Concentration at 1 day . . . . .	476		260		110		29	
Rate on 2nd day . . . . .		1.8		1.1		1.3		0.5
Concentration at 2 days . . . . .	372		197		52		7	
Rate on 3rd and 4th days . . . . .		0.3		0.5		0.5		0.2
Concentration at 4 days . . . . .	337		147		6		0	
Rate on 5th and 6th days . . . . .		0.1		0.6		0		0
Concentration on 6th day . . . . .	332		82		5		0	

We have investigated quantitatively the following phases of glucose utilization:—

*Rate.*—With media containing 100 mg. per cent glucose we have done 10 experiments comprising 40 observations on 24 cultures. Glucose was used initially at rates from 0.5 to 2.0 mg. per 100 mg. wet tissue per day, the rate falling sharply as glucose disappeared.

With a range of higher initial concentrations 7 experiments were done, comprising 64 observations on 29 cultures. Initial rates of glucose utilization were higher, more or less in proportion to concentration, up to 300–400 mg. per cent. Above this level, utilization was at first rapid, but was either greatly slowed or entirely stopped after a few days, while much glucose yet remained. Figures for one such experiment are shown in Table I. The results are in close agreement with those of Gemmill, Gey, and Austrian (8) on Walker rat sarcoma 319.

*Total Glucose Used.*—From a range of higher initial glucose concentrations, total utilization before onset of degeneration similarly appeared to show an optimum at 300–400 mg. per cent, as shown in Table II.

If exhaustion of glucose is prevented by frequent replacement of the regular medium, it continues to be used for a long time and in great amount, especially with daily changes. 2 cultures were followed with daily replacement of medium, 1 for 14 days. This 1 used increasing amounts of glucose each day up to 11 days, totalling 22 mg. of glucose consumed per 100 mg. wet tissue, or an average of 1.6 mg. per day. When cultures were changed every 2 or 3 days so that the media were more nearly depleted, total utilization was less, as shown in Fig. 1.

TABLE II  
*Total Glucose Used from Various Initial Concentrations*

Initial glucose concentration	Glucose used per 100 mg. tissue		
	Experiment 6	Experiment 9	Experiment 7
<i>mg. per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
500–600	4.6	4.0	5.3
350–400	5.1	8.2	5.9
175–200	4.2	4.0	4.4
Duration of experiment, days.....	4	5	6

Figures represent the total glucose which 100 mg. of tissue had used at the end of each experiment.

Exhaustion of glucose may also be prevented by daily additions of concentrated solution without other change in medium, thus maintaining the total concentration near 100 mg. per cent. 9 such cultures had used more glucose at 4 and 6 days than those whose medium was replaced.

*Effect of Colchicine and Phlorhidzin.*—Isolated observations indicate little or no effect of these substances. 1 series of cultures with phlorhidzin and 2 with colchicine were followed.

Colchicine, in concentrations of  $1:10^6$  and  $1:10^7$  did not appreciably alter glucose utilization, although all cells entering mitosis were stopped permanently in metaphase.

Phlorhidzin, in concentrations of 33, 100, and 300 mg. per cent appeared not to prevent the utilization of most of the glucose present, or to be itself destroyed.<sup>2</sup> Phlorhidzin increased the number of mitoses seen in stained cul-

<sup>2</sup> At each concentration of phlorhidzin the level of reducing substances reached a base line corresponding to the amount of phlorhidzin used, and glucosazones could not be obtained in these depleted solutions.

tures. The appearance of the mitoses suggests that it acts through prolonging the mitotic process rather than by stimulating growth. Mitotic counts are seen in Table III.

*Absence of Glucose.*—In explanted tissue not supplied with glucose, degenerative processes are greatly increased. Nevertheless some tissue remains alive for 2 or 3 days. 12 such cultures were followed. At 2 days, they showed a

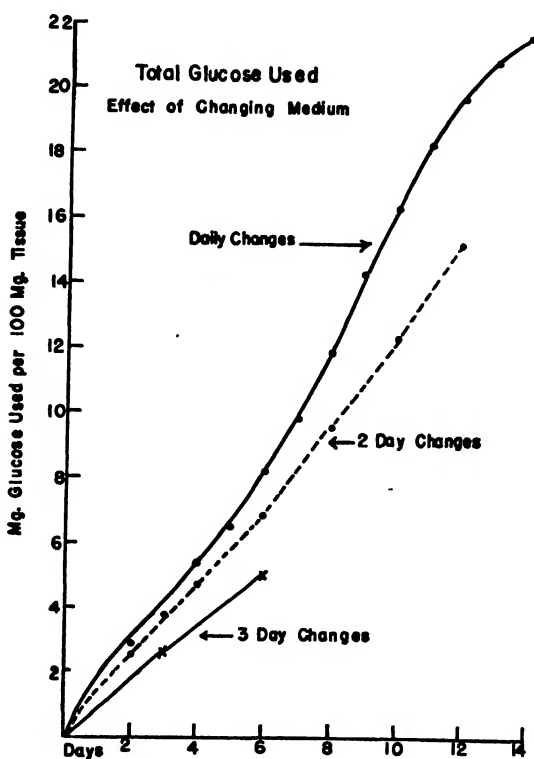


FIG. 1. Cumulative curves showing total glucose used by 1 culture changed daily, and 2 other cultures changed at longer intervals.

slight but definite migration of cells, without mitoses. Most of the tissue, however, degenerated before it began to migrate, and at 4 or 5 days degeneration was complete. Hence metabolic findings, to be discussed later, are of value only if interpreted with caution.

### *Nitrogen Metabolism*

*Amino Nitrogen Production.*—True growth, involving formation of new tissue, must be accompanied by incorporation of protein derived from nitrogenous substances in the medium. Carrel and Baker (9) have shown that

proliferation of fibroblasts is rapid when higher cleavage products of protein are supplied, as by Witte's peptone. If cells do not take up exclusively whole peptone molecules but first hydrolyze some of them to smaller units by means of proteolytic enzymes, an increase of the amino nitrogen of the medium during growth should be seen.

We have followed amino nitrogen production in Baker's solution. 31 observations were made on 26 cultures. The original amino nitrogen concentra-

TABLE III  
*Mitotic Counts on Various Types of Culture*

Medium	Experiment No.	Per cent mitoses				
		Days				
		1	2	3	4	5
Baker's 100 mg. per cent glucose	10	1.3	1.7	0.9		0
Baker's 500 mg. per cent glucose	10	1.6	1.6	1.4		0
Baker's No glucose	18	0			0	0
Baker's No glucose* Added lactate	15		2.0		0	0
Baker's No glucose* Added pyruvate	22	0.7	0.15			
Baker's Added phloridzin 1.0 mg. 0.3 mg.	20		4.3 8.2			
Amino acid medium	14	1.8	2.1	1.4		

\* These cultures showed widespread degeneration. Mitoses were counted in the relatively small areas of healthy cells.

tion was about 14 mg. per cent which is one-seventh of the total nitrogen. This value rose after contact with tissue grown under our standard conditions. Hydrolysis was rapid at first, but decreased markedly in rate after a day or two when the same medium was left on the culture. A maximum rise of 24 to 31 mg. per cent in 6 days was observed under these conditions. Daily change of medium for 3 days appeared to increase the daily production of amino nitrogen.

*Total Nitrogen.*—Utilization of total nitrogen from the medium during growth is difficult to demonstrate in these experiments due to the high protein content of the small amount of plasma used, and to partial autolysis of explants. De-



terminations on cultures grown in nitrogen-free Tyrode solution showed that up to 15 mg. per cent protein nitrogen may thus be added. Total nitrogen values in Baker's solution usually remained quite constant or showed a rise. It therefore seemed advisable to look for utilization of amino nitrogen from a medium whose nitrogen was mainly in this form.

An amino acid solution was prepared by hydrolyzing Witte's peptone with sulfuric acid. This was then used to replace the peptone in a culture medium containing all other ingredients plus 10 mg. per cent tryptophane. The final amino nitrogen content was 65 mg. per cent or about 400 mg. per cent amino acids, which is roughly 4 millimols per 100 cc.

With this medium we obtained excellent growth over 6-8 day periods, with mitotic rates comparable to or exceeding those in the peptone medium (see Table III). Certain differences in behavior from that in Baker's solution were, however, observed. In cultures of embryo muscle a number of giant multinuclear cells were seen using this medium, but never in regular Baker's solution. These cells bear some resemblance to those seen in regenerating adult muscle. The two types of growth are contrasted in Fig. 2. Our amino acid solution was identical with Baker's solution in every possible respect except that the peptone had been hydrolyzed. These effects, therefore, are probably due to specific actions of amino acids, singly or as a group.

We ran 5 experiments using this medium, comprising 60 observations on 29 cultures. In 2 experiments amino nitrogen remained constant for 5-7 days. In 1 case this was associated with tissue degeneration but in the other experiment the tissue appeared in good condition. In the remaining experiments amino nitrogen fell, the tissues grew well, and used glucose at about the same rate as in Baker's solution. Although the variation in results has not been satisfactorily explained, it may be concluded that nitrogen can be progressively used from an amino acid medium. An initial rate of about 0.1 mg. amino nitrogen used per 100 mg. tissue per day dropped to about one-third that value after 2 days. This lessened uptake bears some relation to exhaustion of glucose, for when the glucose level was maintained near 100 mg. per cent, amino nitrogen utilization continued at a somewhat higher rate. Typical results are graphically shown in Fig. 3.

In cultures depleted of glucose the amount of urea plus ammonia nitrogen found accounted for a large proportion of the amino nitrogen lost, whereas a negligible amount of urea plus ammonia appeared when glucose was added. This would seem to indicate that amino acids are deaminated and burned in the absence of glucose, while in the presence of glucose they are stored. Total nitrogen values were somewhat lower in the culture media to which glucose had been added, indicating nitrogen utilization.

#### *Lactic Acid Production*

Previous workers have found extensive lactic acid production by chick fibroblasts. It has been noted that a decreased growth rate resulted in an increased



FIG. 2a

FIG. 2b

FIG. 2. Photomicrographs of embryo muscle cultures. (a) Grown in peptone medium (Baker's). (b) Grown in medium similar to Baker's except that the peptone had been hydrolyzed to amino acids. Stained with hematoxylin.  $\times 80$ .

ratio of lactic acid to size of cultures (Demuth and Meier (10); Meier (11)). About 60 per cent of the glucose used in 48 hours has been accounted for as lactic acid, and glycolysis has been considered characteristic of the chemodynamics of tissue cultures (Krontowski (3)). Pomerat and Willmer (12) offer much indirect evidence that non-phosphorylating glycolysis is essentially linked with growth in cultures of embryo tissues. However, measurements on cultures transferred to Warburg vessels (Lipmann (13, 14)) appear to show that glycolysis is not the characteristic energy source of growth.

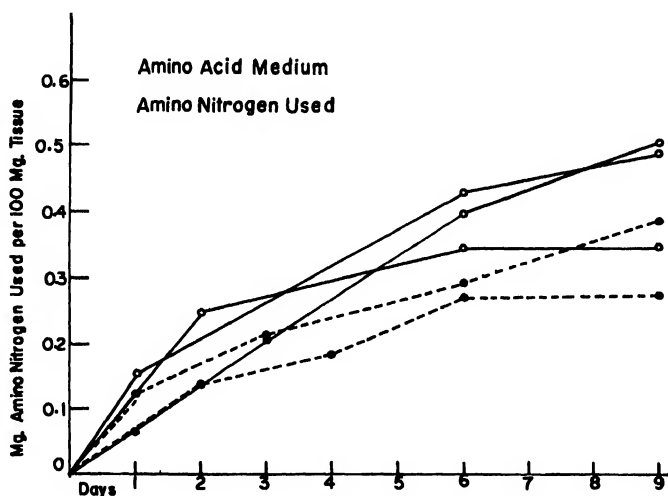


FIG. 3. Amino nitrogen utilization from the amino acid medium. Each curve represents a single culture sampled for analysis at the times shown by points. Resulting changes in volume have been taken into account. Solid lines represent cultures to which glucose was added at 2, 4, and 6 days. Dotted lines represent cultures to which no glucose was added.

Our studies suggest a relation between glycolysis and glucose utilization. They may be divided into six groups.

A. When 100 mg. per cent initial glucose is used up by the tissue: 8 experiments were done, with 32 determinations on 23 cultures. Lactic acid invariably appeared while glucose was falling rapidly, rising during the first 48 hours to values between 25 and 45 mg. per cent. After glucose had disappeared, decreased amounts of lactic acid were found in 3 of these experiments.

B. When initial glucose is 500–600 mg. per cent and is not all used up by the tissue: 5 experiments were done, comprising 17 determinations on 17 cultures. Lactic acid appeared more rapidly than at lower glucose concentration and continued rising to 100 mg. per cent or over in 4 to 6 days.

C. With daily change of medium: in 1 culture given fresh medium and

analyzed daily, from 25 to 42 mg. per cent lactic acid was formed each day for 13 days. Production reached a peak at 10 and 11 days, after which smaller amounts were formed. For the first 11 days 60 to 70 per cent of the removed

TABLE IV  
*Lactic Acid Production under Various Conditions*

Group	Conditions	Ex- peri- ment No.	Constituent	Amount per 100 mg. tissue										
				At start	Days									
					1	2	3	4	5	6	12	13	14	
				mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	
A.	100 mg. per cent glucose	10	Glucose used	0	0.7	1.0	2.4		2.4					
			Lactate	0	0.6	0.7	0.4		0.2					
	100 mg. per cent glucose	31	Glucose used	0		2.1		2.3	2.4					
			Lactate	0		0.9		0.6	0.7					
B.	600 mg. per cent glucose	34	Glucose used	0	5.0				8.4	10.8				
			Lactate	0	1.3				2.1	2.7				
C.	Daily change of medium	33	Glucose used	0	1.7	1.2	1.1	1.7	1.2	1.6	1.3	1.1	0.7	
			Lactate	0	1.2	1.3		1.1	0.9	1.1	1.2	1.1	0.7	
D.	No glucose	18	Lactate	0		0.3		0.4	0.6					
E.	Constant glu- cose	19	Lactate	0		0.1		0.7	2.0					
		29	Lactate	0			2.0		3.3					
F.	Added lactate	15	Glucose used	0		1.5			3.6					
			Lactate	0.7		1.3			0.7					
	Added lactate; no glucose	15	Lactate	0.6		0.8		0.6	0.6					

All cultures were grown in Baker's solution containing 100 mg. per cent glucose, with modifications as shown.

Figures represent total glucose used and total lactate appearing in an unchanged medium except in group C (daily change), where the figures represent daily glucose consumption and lactate formation.

glucose usually appeared as lactic acid. On the 14th day and last day, the proportion had risen to 96 per cent; at this time the cells were healthy but there was extensive liquefaction of the plasma.

D. In the absence of glucose: 1 experiment was done, with 3 determinations on 3 cultures. Lactic acid was formed, but reached only 15 mg. per cent. It is likely that this rise is associated with tissue degeneration.

E. At "constant" glucose level: 2 experiments were done, with 5 deter-

minations on 6 cultures. When glucose was maintained near 100 mg. per cent by daily additions, lactic acid rose progressively.

F. Added *dl*-lactate: 17 mg. per cent was originally included in the culture medium. Determinations on 5 cultures showed that the lactic acid content first rose and then fell to the original level, but not below. This was true both with and without glucose present.

Typical experiments illustrating these findings are shown in Table IV. Results are given in milligrams per 100 mg. tissue in order to make the findings of different experiments comparable.

### *Pyruvate Utilization*

2 experiments comprising 10 cultures and 10 determinations were conducted with 50 mg. per cent pyruvic acid (neutralized) added to the medium, one in the presence of the usual amount of glucose, the other without glucose. The fall in bisulfite-binding values of both media showed rapid utilization of added pyruvate, with values close to zero after 28 hours. Incubation of the pyruvate medium without tissue caused little fall in bisulfite bound, and so it appears that with and without glucose the tissue rapidly disposes of pyruvate. In the absence of glucose, pyruvate allowed slight but definite growth to occur, as with lactate. This growth was much less than that seen with a corresponding amount of glucose, which is confirmed by the mitotic counts shown in Table III.

### DISCUSSION

To understand these results we must consider carefully the test object. The life of a tissue culture includes four periods whose chief attendant features are: (1) injury of removal, (2) tissue growth, (3) regressive change, retardation of cell division, and visible alterations such as vacuolation, and (4) degeneration and autolysis.

In the period of injury we have largely intact cells plus those which have been cut or injured so that they die. The injured cells may reach 50 per cent of the amount explanted (15). Cell contents, autolytic products, and intracellular enzymes are thus added to the medium. This probably continues for a few hours under normal conditions, after which the metabolism of the culture is that of intact growing cells.

Thus we are dealing with the activity of a complex physiological system similar to that in the whole animal. The substrate is outside an intact cell surface, and localization of enzyme systems within the cell is preserved. In tissue extract experiments catalyzed reactions are occurring in a single phase system without cells, while in tissue slice experiments observations are made during the period of injury and under conditions precluding recovery and growth. Hence study of tissue cultures during the period of growth gives a more faithful picture of metabolic reactions in intact cells.

It is to be expected that an unchanged medium loses efficiency by loss of essential constituents and by accumulation of end products. The increased life of cultures bolstered by additions of glucose to an otherwise unchanged medium indicates that glucose loss may be a limiting factor in this regard. Daily changes of the whole medium increase the life span of cultures still further.

The amount of glucose used appears from our observations to have no direct relation to growth. Cultures treated with colchicine in concentrations which block all mitoses continue to use glucose at the usual rate. Moreover cultures grown in 500 mg. per cent glucose use more than those grown simultaneously in 100 mg. per cent, but examination of the fixed tissues at various periods shows about the same area of migration and closely similar mitotic counts in both groups. This observation appears to be in contrast to those of some previous workers whom we have quoted; the difficulties involved in estimating true growth in cultures must be emphasized. Probably the energy requirements for life and growth are easily supplied, and higher amounts of glucose do not increase growth rate because it is already limited by factors such as permeability, rate of intracellular synthetic reactions, or availability of essential foodstuffs. The enzyme systems for disposal of glucose appear to be stimulated to greater activity, possibly by a simple mass law effect. Conversion to glycogen, either in muscle or liver, has not been demonstrated by staining methods in our cultures. Presumably more energy is evolved and somehow absorbed when the rate of glucose removal is increased.

Glycolysis appears to be associated with the period of growth, for lactic acid production was related in rate and amount to glucose utilization. Lactate is also produced during the period of regressive change. While the amount evolved is less than in a healthy culture, it tends to represent a greater proportion of the total metabolism. The small amount of lactate formed in cultures not supplied with glucose increased with time and with the onset of degeneration.

Our experiments afford some evidence that tissues can utilize lactate after their glucose is used up. At this point lactate values frequently showed a tendency to fall from levels reached earlier in the life of the culture. When *D*-lactate was added to cultures without glucose no loss was observed, but microscopic observations showed more migration and less vacuolation with than without lactate. Moreover many mitoses were seen after 2 days when control cultures showed no dividing cells (see Table III) indicating that in cultures deprived of glucose lactate favors cell division although it is little used. Degeneration was delayed for about 2 days by added lactate. Pomerat and Willmer (12) report similar findings in cultures of osteoblasts without glucose to which lactate was added.

Lactate found in cultures without glucose may originate from glucose formed from certain amino acids. In support of this hypothesis is the fact that in an

amino acid medium without glucose tissues produced over ten times as much urea plus ammonia nitrogen as those kept supplied with glucose. Deamination of certain amino acids prior to their conversion to glucose would explain this difference.

Although utilization of nitrogen must obviously occur during growth, the quantitative demonstration of this process when amino nitrogen is practically the only available form of nitrogen is of special interest. There has been considerable question as to whether cells in culture could make use of nitrogen supplied as amino acids, although Vogelaar and Erlichman (16, 17) believe this may be possible under optimal conditions.

The definite morphological stigmata of cells grown in our amino acid medium indicate that the medium affects certain processes differently from a peptone medium. Vogelaar and Erlichman believe that amino acids may be preferentially adsorbed on the cell surface to such an extent as to interfere with normal processes. In this case morphological changes may result from specific effects of the amino acids upon the cell surface. It must be considered, however, that these variations may be explained by an altered metabolism within the cell: in the case of a peptone medium nitrogen may be taken up in the form of peptides and utilized as such, while amino acids taken up directly might follow a different course. Although our experiments have not yielded proof of the utilization of nitrogen in peptide form, morphological observation suggest this possibility.

#### EXPERIMENTAL

*Planting Technique.*—Tissue to be explanted was removed from the embryo under the usual sterile conditions and placed in small, weighed, sterile Petri dishes. It was then cut up and weighed to the nearest 0.1 mg. as rapidly as possible. Tyrode solution was then added and the tissue transferred to the inside surface of a roller bottle previously coated with a thin film of plasma, which formed a holding clot when in contact with the tissue. Care was taken to introduce all the weighed tissue into the bottle, and to remove the entire excess of plasma and Tyrode solution used in the transfer before adding the culture medium. Thus a known wet weight of tissue was explanted.

*General Procedure.*—Bottles were placed in the roller apparatus in an incubator at 37°C., removed serially at intervals up to 7 or more days, and the entire fluid decanted for analysis. Thus a progressive picture of events was obtained. For some determinations it was possible to sample one bottle at intervals without greatly changing the volume. Results obtained in this way showed great reproducibility, especially in the case of glucose utilization.

All cultures were examined microscopically every day. This necessitated no interference with their environment except when media were sampled or changed. Areas of fibroblastic and epithelial migration were qualitatively noted, as well as the extent of changes such as rounding of cells, vacuolation, liquefaction, or focal or generalized degeneration. The migration rate of cells was estimated when desired by measure-

ments on camera lucida drawings of explants. At the end of each experiment the cultures were fixed, the coverslip was removed by immersion in acetone, and the tissue was stained with Harris' hematoxylin and mounted. Microscopic examination was then made and in certain cases mitoses were counted in groups of 1000 peripheral cells. Typical mitotic counts are given in Table III.

Ordinarily, tissue cultures under standard conditions grew normally for at least 3 days with mitoses decreasing on the 3rd day. After 5 days mitoses were usually absent in unchanged media, and rounding or vacuolation had begun, but little frank degeneration occurred until later. During the period of most active growth the daily radial increase was 0.5 to 0.8 times the radius of the explant, and about 1.5 per cent of the cells were in mitosis.

*Analytical Methods.*—All determinations of changes in composition of media must of course be controlled by parallel determinations on original media. This is especially true since observed values for some metabolites in Baker's solution differ from those in solution in water. A substantial non-glucose-reducing value is found which is not diminished by tissues in the absence of glucose, and for which corrections must be applied. Interfering substances also give values for lactate, and affect lactate determinations so that special modifications are necessary. The metabolites listed below were determined by the following methods.

*Glucose.*—Folin's micro method using potassium ferricyanide reduction, as adapted for the Evelyn photoelectric colorimeter.

*Total Nitrogen.*—Kjeldahl digestion and distillation followed by titration with 0.02 N alkali.

*Amino Nitrogen.*—The Van Slyke gasometric procedure, using the manometric gas apparatus.

*Ammonia Plus Urea Nitrogen.*—The manometric sodium hypobromite method of Van Slyke (18).

*Lactic Acid.*—The gasometric procedure of Avery and Hastings (19), using the Van Slyke manometric apparatus, was first employed. For reasons of greater specificity and convenience our later determinations have been done by Edwards' modification of the aeration method of Friedemann, Cotonio, and Shaffer (20, 21). We further, altered the procedure partly according to the suggestions of Friedemann and Kendall (22) for peptone media, using 0.06 M phosphoric acid and 0.025 N potassium permanganate. Each of these methods was carefully developed as to time of reaction, etc., to give nearly quantitative recovery of lithium lactate added to Baker's solution, and substantial agreement was obtained when both methods were applied to the same solution. Even so, our results are indicative of changes in lactate rather than true values, and are probably accurate to not more than  $\pm 10$  per cent.

*Pyruvate.*—The bisulfite-binding method of Clift and Cook (23).

*Preparation of Amino Acid Solution.*—Witte's peptone was hydrolyzed with 20 per cent sulfuric acid until amino nitrogen had risen to an almost constant level of 78 per cent of the total nitrogen. Barium hydroxide was added to pH 7, and the precipitate of barium sulfate filtered off and washed. The filtrate was again carefully adjusted with sulfuric acid and barium hydroxide to the lowest obtainable content of both barium and sulfate ions. The final filtrate was concentrated as far as possible without causing precipitation of insoluble amino acids.



## SUMMARY

1. The metabolism of chick embryo tissues has been followed by analysis of the culture media after various periods of incubation in roller bottles.

2. The initial rate of glucose utilization is increased by increasing glucose in the medium from 100 to 500 mg. per cent. Total glucose used can be increased in the same way or by daily addition of small amounts. Glucose is used in greatest amount when the medium containing 100 mg. per cent is replaced daily.

3. Although glucose consumption appears necessary for survival of cultures it may be used at a rate far in excess of that required for life and maximal growth. Complete blocking of mitosis by colchicine does not alter the rate of glucose utilization.

4. Proteolytic activity of the cultures is shown by an increase in the amino nitrogen of the peptone medium after incubation with tissue.

5. Utilization of nitrogen from an amino acid medium is shown by a decrease in the amino nitrogen of this medium. Cells obtaining their nitrogen from amino acids proliferate as rapidly as those grown in a medium identical except for the substitution of peptone, but the cell type is markedly different, in that embryo muscle forms cells resembling regenerating adult muscle.

6. Lactic acid was formed in both the presence and absence of glucose. Its formation increased with increased glucose utilization. There is some evidence that lactate may be utilized, and that it favors growth in the absence of glucose.

7. Added pyruvate was rapidly metabolized by the tissues. It, too, favors growth slightly in the absence of glucose.

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# THE INFLUENCE OF DETERGENTS ON SOME PHYSIOLOGICAL PHENOMENA, ESPECIALLY ON THE PROPERTIES OF THE STELLATE CELLS OF THE FROG LIVER\*

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## *Introductory Remarks about Some Physicochemical Properties of Detergents*

The detergents are a special group of nonpolar-polar surface-active organic electrolytes. They reduce the interfacial tension not only between water and air, but also between water and certain organic liquids. Their molecules are composed of two portions, one polar hydrophilic, which has a tendency to anchor the molecule to water, and one nonpolar hydrophobic organophilic, which is attracted towards the non-aqueous phase. The result is a molecular orientation at the interface due to the opposing forces. These can be, more or less, in balance according to the strength of the alternative affinities. In general, an increase of hydrophilic affinity in the interfacial array of the nonpolar-polar molecules will lead to a stronger pull on the nonaqueous phase toward the water and may have a disintegrating or a dispersing effect. Stronger organophilic properties on the other hand will favor a wetting action; *viz.*, the fixation of a film of water on the nonaqueous phase or on its separate components. Regarding especially the interface between a cell and its surroundings or the intracellular interfaces, hydration, swelling, and dissolution can be due to the wetting action on the colloidal structures or the micellae (Katz, 1933-34; von Kuthy, 1931; Höber and Moore, 1939). Protein molecules are unfolded or disrupted by the pull and thus denatured (Anson 1939; Mirsky, 1938). In conjugated proteins the bonds between the protein part and the prosthetic group are severed (Anson, 1939; Kuhn, 1940), viruses and enzymes are inactivated, and the final result of such effects on cells is cytolysis.

However, cytolysis is brought about also by other chemical means. Inorganic ions, including H and OH, produce cytolysis chiefly by their influence on proteins, either by changing their ionization and consequently their attraction of water dipoles

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The detergent compounds used were kindly supplied by E. I. du Pont & Company, by the American Cyanamid Company, and by Riedel-de Haen, Inc.

or by changing their solubility and forming precipitates. A third group of substances significant for their cytolytic power are the anesthetics. Though belonging to most divergent classes of chemicals, they are united into one group by their common physicochemical property of being soluble in "lipoids." Some of them are devoid of polar groups, (e.g. hydrocarbons, halogenated hydrocarbons, ethylchloride, ether); they fail to reduce the interfacial tension between water and an organic liquid (olive oil, benzene) (Lazarew, Lawrow, and Matwejew, 1930; Meyer and Hemmi, 1935). Their cytolytic power, which, in general, appears only in higher concentrations, probably can be accounted for mainly by their affinity for the cell lipoids. Other anesthetics have a moderate nonpolar-polar molecular structure, (e.g. alcohols, ketones, amides), and, due to this property, they lower the interfacial tension. In this respect they resemble the detergents. However, the detergents are strong or fairly strong electrolytes, the anesthetics are nonelectrolytes. This important difference may be explanatory of the characteristic effects of the detergents, in which not only the opposing van der Waals forces, the hydrophilic and the hydrophobic affinities are involved, but also electrostatic forces. In other words, anesthetic power can be exhibited by nonpolar or by moderately nonpolar-polar nonelectrolytes, detergent power by nonpolar-polar electrolytes.

So far there has not been much study concerning the physiological effects of detergents, except the bile salts, which also are nonpolar-polar electrolytes. This paper will deal chiefly with bile salts, with higher sulfated alcohols, and with alkyl esters of sulfosuccinic acid.

#### *Correlation between the Surface Activity and the Cytolytic Power of Detergents*

The surface activity of the detergents was measured by the drop count method, the lowest concentration being determined, which gave a value different from water. The cytolytic power toward red blood corpuscles was determined by mixing 0.1 ml. of beef blood and 10 ml. 0.9 per cent NaCl solution containing the detergent in concentrations decreasing stepwise to zero. During and after sedimentation of the red cells at room temperature it was observed, at what concentration after several hours the supernatant fluid just appeared colored by hemoglobin or remained colorless. In order to measure the cytolytic power of the detergents towards muscles, it was found adequate to ascertain the minimal concentration, which produces an injury potential, when one end of a sartorius muscle is in contact with Ringer, the other with Ringer plus detergent. Whereas hemolysis such as evidenced by the escape of hemoglobin is irreversible, the myolytic method allows one to study in how far the potential changes by their reversibility may be indicative of alterations of the muscle fibers comparable to those changes, which, in a previous study, have been considered analogous to normal physiological events associated with muscle and nerve activity (Höber *et al.*, 1939).

In this way approximate threshold values for a number of detergents were obtained. In Table I they are listed as molar concentrations.

It is obvious from the table that the threshold values for the surface activities and the cytolytic activities, on the whole, run parallel to each other, as could be expected. But what was unexpected is that the numerical values for these activities are in as good agreement as they really are shown to be by our experiments, although the threshold change in the molecular arrangement at the interfaces air-water, muscle-water, and red cell-water is measured, respec-

TABLE I

*Threshold Concentrations in Mol./L  $\times 10^4$  of Surface, Hemolytic, and Myolytic Activity of Some Detergents*

Substance	Molecular weight	Increase of drop number	Hemolysis		Injury potential concentration and reversibility	Activation of stellate cells
			Threshold concentration	Sub-threshold concentration		
Alkyl sodium sulfates (du Pont)						
Dodecyl	288	1	0.7	0.25	2.4 +?	1
Lauryl	274	3	0.8	0.5	3	
Decyl	260		3	2.5	10 +?	1-5
Octyl	232		45	30	80 +?	
Dialkyl sodium sulfosuccinates (Amer. Cyanamid Co.)						
Dioctyl (Aerosol OT)	444	0.7	0.7	0.3	0.7 +	5
Dihexyl ( " MA)	388	12	13	3	16 +	50
Diamyl ( " AY)	360		17	15	14 +	
Dibutyl ( " IB)	332	~ 60	~ 75	60	75 +	
Bile salts						
Sodium taurocholate	537		10	5	20 +	0.3-0.6
Sodium glycocholate	487		10	5	20 +	0.3
Sodium dehydrocholate	424	No effect	100 (no effect)		100 (no effect)	100 (no effect)

tively, by the change of the interfacial tension, the change of the permeability of a plasma membrane to ions, and the change of the permeability to hemoglobin. Furthermore, in the table is shown the significant fact that the development of the injury potentials is a reversible process; *viz.*, that upon substituting plain Ringer for the detergent solution, the injury effect disappears. However, the recovery takes a longer time or it requires rinsing the muscle more than once, when the alkyl sulfonates have been applied instead of the alkyl sulfosuccinates or the bile salts. This recovery can be considered—generally speaking—as a rearrangement of the molecules in the interfacial layer, when, caused by a stronger or a weaker attachment of the nonpolar

C-atom chains, the membranes are subjected to dispersing or to hydrating forces of variable magnitude. This will be discussed in more detail in a following paper.<sup>1</sup>

*Effects of Various Concentrations of Detergents upon the Microscopic Appearance of Stellate Cells*

**The Problem.**—In this paragraph a more detailed study of the influence of some detergents upon one particular system of active cells, the Kupffer cells or stellate cells of the liver, will be described. The starting point is the following group of observations. The isolated frog liver, when perfused with Ringer solution with the addition of a very small amount of dye, has been found to excrete in the bile a great number of various dyestuffs; several hundred or even more than a thousand times concentrated these appear for hours in a cannula fixed in the gallbladder (Höber and Titajew, 1928; Höber, 1939). In contrast to this process, under the same experimental conditions the liver loses its well known capacity to store in its stellate cells fine suspensions and colloidal material, but regains it after the addition to the perfusion fluid of a small amount of serum (Höber, 1940). There are several interpretations, as will be discussed later. This study was chiefly suggested by the work of Clark (1913) on the "hypodynamic state" of the frog heart, which loses its activity after being perfused for a few hours with Ringer solution, and which can be reactivated by the addition of small amounts of serum. In a thorough investigation this beneficial action of serum was shown by Clark to be due not to its proteins, but to its small content of lipoids, to lecithin, and to soaps of the higher fatty acids ( $C_{10}$  to  $C_{18}$ ), briefly, as we may say now, to substances belonging to the class of nonpolar-polar organic electrolytes. The reviving effect then may be thought of as being based on a slightly dispersing influence upon the cellular interfacial structures, which, when elicited with stronger and stronger concentrations, ultimately passes over from a beneficial effect into a disintegrating cytolysis. Correspondingly, the inactivation of the stellate cells by Ringer perfusion and the reactivation by serum may be looked upon as a hypodynamic state and its reversal. This hypothesis has been tested experimentally.

**Method.**—The perfusion cannula was fixed in the abdominal vein at the inside of the body wall, after the vessels going to and coming from the liver had been blocked

<sup>1</sup> To what extent the effects of the detergent agents must be referred to the surface membranes of the cells or to the intracellular phase boundaries, is unknown. Many detergents, e.g. the alkyl sulfosuccinates, though being strong electrolytes in dilute solution, are highly soluble in organic liquids. Aerosol OT, for instance, is soluble in many polar and nonpolar solvents. Therefore, supposedly, their relative lipid solubility also is high (see American Cyanamid Company, 1941).

by a mass ligature around cardia, porta hepatis, radix mesenterii, and rectum. The perfusion fluid,  $\text{CO}_2\text{-HCO}_3$ -buffered Ringer solution (pH 7.5), entered under a pressure of 8 to 10 cm.; the outlet was provided by cutting the sinus of the heart. The rate of perfusion was controlled throughout the experiment and was kept fairly constant by a screw clamp.

More than 350 experiments on *R. pipiens* (mostly females) were performed, mainly according to the following scheme: (1) For 30 minutes Ringer perfusion. At the end of this period the liver appeared to be more or less blood-free. (2) For 10 minutes Ringer with (or without) 2 per cent beef serum. (3) For 45 minutes the same plus 0.02 per cent trypan blue Grübler (Akatos) or soluble blue R (du Pont) and, eventually plus a test substance. (4) The three lobes of the liver, usually cut into small pieces placed in formalin and after 48 hours sectioned with the freezing microtom.

In general, two to four frogs of the same batch, of rather the same weight and living under the same conditions, were compared. In order to exclude the effect of differences in the activating power of the serum, the same serum was always applied to all the frogs of a given group.

During the third period the liver turns blue, but often in the different lobes to a different color intensity. In general, slices of the corresponding lobes were compared in the microscopic investigation.

In the majority of experiments, the classical dyestuff for intravital staining of the stellate cells, trypan blue, was used; only after recent failure, for unknown reasons, to reproduce the customary staining effect with new samples of the dye, we substituted successfully the trypan blue by the likewise colloidal soluble blue R.

## RESULTS

### *A. The Microscopic Picture in General*

The picture of a normal frog liver displaying an active system of stellate cells, as gained by this method, is briefly and on an average as follows: The slices show the striking pattern of a dark blue network covering more or less uniformly the entire area of the slice, the meshes being filled mainly by the hepatic cells, and to a smaller extent by the empty lumina of the blood capillaries. The net seems more or less distinctly attached to the capillary walls and it looks as though composed of the homogeneously stained and irregularly shaped stellate cells, which partly project into the capillary lumina, whereas the islets of the genuine hepatic cells, which fill the meshes, appear colorless. Often along the capillary walls, where they are not occupied by the stellate cells, fine dark blue border lines are visible, possibly the flattened endothelium cells.

But, as mentioned before, a picture like this fails to appear after perfusion with plain Ringer solution. On the other hand, due to several causes, even after a succeeding perfusion with Ringer plus serum the liver exhibits the network pattern in very non-uniform intensity of color. Most evident is the seasonal influence. Unlike the capacity of the liver to transport dyestuffs from a low concentration level in the perfusion fluid to a high level in the bile ducts, which, in general, can be demonstrated throughout the year, the system



of stellate cells is more or less at rest during the winter months. But besides this regular periodicity, there occur irregularly gross or fine variations in the staining intensity. These, obviously, are indicative of differences in the activating potency of the sera, probably due to changes in the percentage of the nonpolar-polar substances mentioned before. It was for this reason, that the use of the same serum in a group experiment was necessitated and that control experiments were designed to show that a positive effect was due to the presence of a certain active drug and not to the serum. Thus, the microscopic sections sometimes did not show more than a gossamer network and small pale cells. Stronger effects were indicated by more color and by larger cell bodies, which often had an irregular shape and sometimes long processes. After a detergent had been perfused, the enlargement of the cells could approach pathological magnitude and even end in cytolysis as manifested by protoplasmic desquamated masses extending into the wider capillaries. This terminal state was often marked out as pathologic by the appearance of nuclear staining in the hepatic cells.

### *B. Alterations of the Microscopic Picture caused by Detergents*

1. *Sodium Salts of Conjugated Bile Acids (Glycocholate and Taurocholate).*—The bile salts are known to be the most powerful cholericics. In our experiments they have been found also the most reliable among the detergents tested for producing an increased uptake by the stellate cells of the aforementioned colloidal dyestuffs. The bile salts were applied both as chemically pure preparations (Riedel-de Haen) and as commercial drugs (Kahlbaum, Merck). The optimal concentration was found to be  $0.3$  to  $0.6 \times 10^{-4}$  molar. Among twenty-one double experiments, comparing the effect of Ringer-serum and of Ringer-serum plus bile salt, within this concentration range, eighteen resulted in an increase of the staining effect. That is to say, the network was more distinct and was spread more generally over the area of the slices than in the controls. Subsequent to an increase of concentration up to  $3$  and  $7.5 \times 10^{-4}$  molar there appeared a coarse network filling rather to completeness the capillary spaces; often the single stellate cells appeared to be swollen. At about  $30 \times 10^{-4}$  much nuclear staining was observed, which extended more or less into the center of the islets of hepatic cells; debris of the stellate cells reached the larger vessels. Of great significance is the observation that in the absence of serum the bile salt appeared to be unable to promote the uptake of the dye.

2. *Dehydrocholate (Decholin Sodium).*—This substance is superior to the conjugated bile salts as a cholericic so far as the bile volume is concerned, but it differs from them in that it decreases the content of the main characteristic bile components; it is therefore classified as a hydrocholericic agent. Its effect is associated with a marked increase of blood flow in the hepatic artery (Grodins, Osborne, Ivy, and Goldman, 1941). We have studied the influence

upon the stellate cells, but up to a concentration of  $10^{-2}$  molar no effect was detectable. This can be correlated with the fact that decholin is neither surface-active nor has it a hemolytic or a myolytic power (Table I). The lack of a nonpolar-polar character probably can be accredited to the presence in the molecular ring system of three keto groups.

3. *Alkyl Sodium Sulfates*.—Two members from this series of detergents have been studied, the dodecyl and the decyl compound. The concentrations producing a small, but definite effect have been found to be around  $1 \times 10^{-4}$  and  $1$  to  $5 \times 10^{-4}$  molar respectively. At a concentration of  $10 \times 10^{-4}$  nuclear staining takes place in many hepatic cells. In two experiments this effect diminished from the periphery of the cell islets to the center.

4. *Diocetyl and Dihexyl Sodium Sulfosuccinate (Aerosol OT and MA)*.—The threshold of the augmentation of the dyestuff uptake was found with OT near  $0.11 \times 10^{-4}$  molar, with MA near  $1.3 \times 10^{-4}$ . With OT and with MA at about 10 times higher concentration much nuclear staining appeared. With  $15 \times 10^{-4}$  MA the stellate cells showed disintegration.

5. In previous experiments concerning the capacity of the Ringer-perfused liver to discharge dyestuff in an amazingly high concentration (page 708), it was observed that this power can be reversibly depressed or extinguished by substituting for  $\frac{1}{8}$  of the Ringer solution an equal part of an isotonic solution of certain nonelectrolytes (disaccharides, hexoses, pentoses, polyhydric alcohols, amino acids) and certain organic electrolytes, particularly the salts of mono- and dicarboxylic acids (malonate, succinate, glutarate, malate, fumarate, gluconate, lactate) (Höber and Moore, 1939; Valdecasas, 1931; Höber, 1932). We have tried to determine whether the same inhibitory effect can be demonstrated with the stellate cells. For this purpose we have chosen two substances which were found to be regularly and vigorously effective in the dye secretion, namely, sucrose and succinate. In eight experiments pairs of livers were perfused with Ringer plus serum plus  $0.3 \times 10^{-4}$  molar taurocholate and to one of each of these pairs sucrose was added. In seven of these experiments there appeared a clear cut diminution of the staining effect; *viz.*, the blue network was distinctly finer, compared to the control liver. In two experiments with  $0.11 \times 10^{-4}$  molar Aerosol OT the same result was obtained. On the other hand, we failed to get an unambiguous response with succinate; three experiments were positive, four negative.

#### DISCUSSION

The experiments described have provided evidence that in the incorporation of colloidal dyestuffs by the stellate cells a prominent factor is the presence of serum and that this activation is increased by the addition of nonpolar-polar organic electrolytes, like detergents including bile salts. Increase of activation is equivalent to a greater staining effect indicated not only by the depth of

color, but also by the enlargement of the cells. A considerable enlargement, as brought about by higher concentrations of detergents, is doubtless a pathological appearance, which finally ends in cytolysis. But, is there reason to assume that threshold staining effects can be correlated with a physiological functional activation of the stellate cells,—although apparently the microscopic method is rather inadequate in proving that a certain microscopic picture is the effect of a certain composition of the perfusion fluid? Let us recall that threshold changes were judged only by comparing slices of pairs of livers, one affected by a certain change in the composition of the perfusion fluid, the other the control, or one influenced by a lower, the other by a higher concentration of a drug. After having averaged in this way the threshold changes, how can we answer the question as to whether such changes can be regarded as signifying a greater functional activity or the beginning of death? Certainly, we can recall the fact, that it is a common characteristic of living things that substances, which are stimulants in small doses, are deleterious in higher ones. In previous studies concerning the influence of nonpolar-polar substances upon the dyestuff secretion of the liver (Höber and Moore, 1939; Höber, 1940) we have observed that a certain minimum concentration of a detergent, which has been found prevailing to increase the dyestuff transport, may also display the opposite influence. Such finding emphasizes the fact, that it can be essentially difficult to classify a phenomenon as still physiologic or as already pathologic. In order to avoid this dilemma as far as possible it is desirable to show that a threshold effect is a reversible process. Therefore, in our work we have laid stress upon showing that the injury potential, which can be brought about by powerful cytolytics such as our detergents, if cautiously applied in minimum concentrations and for a short time can be decreased, eventually to zero, by rinsing the muscle with Ringer. But, as indicated in Table I, there are variations in the ease of reversibility, and a question mark means that recovery was brought about only slowly and after the injured end of the muscle had been flooded repeatedly with Ringer (see also Höber *et al.*, 1939). Furthermore the reactivation of the hypodynamic heart in the experiments of Clark (page 708) may be mentioned, although as yet only a few nonpolar-polar substances which are normal components of the blood serum, have been shown to be effective. Finally, it may be recalled that 30 years ago Loeb found that surface-active agents, among them bile salts and higher fatty acids, administered to unfertilized sea urchin eggs induced cleavage and development, whereas higher concentrations caused cytolysis.

Certainly, it would be most desirable in this connection to give direct proof that the threshold staining effect on the stellate cells is reversible. A slight contribution toward such proof is furnished by the observation of the antagonism of sucrose to the action of detergents (see page 711) so strikingly shown in earlier experiments on dyestuff secretion by the liver. This experiment was

conducted for the following reason: it has been shown previously that a large group of organic nonelectrolytes and of organic anions, both of them hydrophilic because of the presence of a relatively great number of OH and of COOH radicals in their molecules, exhibit an antidispersing and dehydrating and shrinking effect upon hydrated colloids and have vigorous inhibitory but reversible influence on the capacity of the liver to secrete dyestuffs. In addition there is another group of organic substances, nonpolar-polar, *viz.*, half organophilic and half hydrophilic, which is dispersing and therefore cytolyzing, and which favors dyestuff secretion. This difference in distribution of physico-chemical properties suggested the possibility of selecting pairs of substances, which might antagonize each other in their influence upon the dyestuff secretion. In such experiments, as mentioned before, sucrose gave the expected effect, succinate did not. The reason for the latter result is unknown.

Now, the question arises as to how to explain the favorable influence of serum upon the uptake of dyestuff. In this connection an important point is that, in contrast to the stellate cells, the secretory epithelia of the liver and of the tubular walls of the kidney are able to transfer dyestuffs from the blood vessels into and across their own substance whether serum is present or not. Evidently, the principal mechanisms of intake are different. Here it is suggestive to consider the well known phagocytic properties of the stellate cells and other representatives of the reticulo-endothelial system, which enable them to incorporate microscopic as well as submicroscopic particles, among these hemoglobin and other proteins. For several reasons, the latter can be assumed to form a film around the particles of the colloidal dyestuffs, thus predisposing the dyestuffs to adhere to the cell surface. However, this cannot be more than one factor in explaining the activating power of serum, since the detergents also must be taken into consideration. Perhaps, it is adequate to assume that the phagocytic mechanism cannot play its part, unless protein is present at the surface of the stellate cells. Two observations mainly can be cited in support of such a concept. As mentioned before, in the absence of serum the detergents, even the most reliable ones, the bile salts (page 710), are unable to stimulate the uptake of the colloidal dye into the stellate cells (page 710). Under these conditions one finds the dye, especially soluble blue R, lined up along the walls of all vessels, not only the walls of the capillaries encircling each islet of the hepatic cells, but also the walls of the larger vessels, their inner sides being covered by a fine blue coat, and the stellate cells remaining fairly invisible. This distribution of the dye is entirely different from that appearing subsequent to perfusion with the addition of the 2 per cent serum. The regular lining all around the islets and the regular dark contour on the inner wall of the larger vessels fail to be formed. Instead, the stellate cells, activated by the detergent to a heavy incorporation of protein, increase in volume and absorb with the protein a great load of dyestuff. In this way, on

the one hand, the dyestuff is diverted from the walls of many vessels. On the other hand, it is shifted to and accumulates in the enlarged stellate cells, which project into the capillary lumina and thus form the characteristic coarse network, as described above (page 710).

Finally, one more feature in the microscopic picture of the stained stellate cells seems worth discussing. In our experiments, the stellate cells in general appeared homogeneously stained, whereas ordinarily they are described as containing the trypan blue in fine dark blue granules.<sup>2</sup> Possibly our pictures could be interpreted as being due to extensive impairment of function caused by the artificial perfusion of the isolated organ. However, rather the contrary may be true. The appearance of granules in cells stained with sulfonic acid dyestuffs such as those used in our experiments, in general is suspicious of a pathological condition of the protoplasm. As a matter of fact, the normal appearance of living cells, while absorbing and transporting dyes, is that of a homogeneous solution. Only when a living cell is overloaded by excess dyestuff in the medium, does the formation of granules occur. This concept of the formation of granules is corroborated in a very convincing way by the observations of Chambers and Kempton (1933), regarding the secretory activity of the kidney tubules in explants of the mesonephros of chick embryos. In our own experiments, dyestuff transfer and accumulation in the secretion of the frog's kidney and liver was followed over hours without displaying any sign of the dye being segregated in granules (see further Höber, 1935). The customary procedure for studying active stellate cells was and is to inject trypan blue and to observe the microscopic appearance not earlier than 1 or 2 days later. With this procedure one gets the conventional picture with frogs. However, studying the microscopic distribution of the dye only a short time after its intravenous injection yields prevailing the homogeneous coloration, as it appeared in our perfusion experiments.

#### SUMMARY

1. After a consideration of the physicochemical properties of detergents, it was deemed worth while to study some of their physiological effects. As nonpolar electrolytes, the detergents are surface-active and as such cytolytics; but probably due to their dispersing and wetting properties, they are cytolytic in a fashion different from that of other cytolytics. The detergents tested were alkyl sulfonates, alkyl sulfosuccinates, and bile salts.

2. The cytolytic power has been tested in two ways, (1) with red cells by following the escape of hemoglobin, (2) with muscles by measuring the development of an injury potential. In both series of experiments the threshold concentrations of action have been determined. The effect on the potentials has proved to be, in general, reversible.

<sup>2</sup> See, e.g., the colored plates in the article by Cappell (1929).

3. The hemolytic and the myolytic power run fairly parallel to the surface activity.

4. Dehydrocholate has been found to be lacking in nonpolar-polar properties.

5. The stellate cells (Kupffer cells) of the Ringer-perfused frog liver are unable to take up colloidal dyestuffs (trypan blue and soluble blue R), except after addition of a small amount of serum to the perfusing Ringer solution. Only under the latter conditions, the uptake of dye is increased by adding a detergent. This seems to be due to the combined action of the proteins and the detergents.

6. The effect of relatively high concentrations of detergent is disintegration of the stellate cells; *viz.*, cytolysis. There are reasons to assume that small concentrations, which produce a threshold increase of the dyestuff uptake, raise the functional activity.

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## STUDIES ON CELL METABOLISM AND CELL DIVISION

### VI. OBSERVATIONS ON THE GLYCOGEN CONTENT, CARBOHYDRATE CONSUMPTION, LACTIC ACID PRODUCTION, AND AMMONIA PRODUCTION OF EGGS OF *ARBACIA PUNCTULATA*

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In the course of an experimental program dealing with the question of the relation of respiration to energy transfer for cell division in *Arbacia* eggs, it has been reported, in previous papers of this series, that these eggs contain a number of substances which are known to be concerned in the oxidative breakdown of carbohydrate in animal cells.

To facilitate evaluation of the quantitative importance of various enzyme systems in the metabolism of the living egg it was important to establish, under conditions exactly comparable to those used for determination of the components of the respiratory chain in *Arbacia*: (a) the amounts of various types of carbohydrate present in the eggs, and especially the fraction of this carbohydrate which was in the form of glycogen and possibly subject to the sequence of metabolic reactions which begins with the phosphorylation of glycogen (1), and (b) the extent to which carbohydrate was used as a metabolic substrate at various stages of egg development.

Confirming the observations of Perlzweig and Barron at Woods Hole during the season of 1927 (2), it has been found that *Arbacia* eggs contain a considerable amount of acid-hydrolyzable carbohydrate, but no free reducing sugar. In confirmation of the unpublished experiments of Blanchard (3) which were also performed during the season of 1927, it has been found that *Arbacia* eggs contain an alkali-stable, alcohol-precipitable carbohydrate. In extension of previous work, it has been found that this substance, which corresponds in its color reactions, yield of reducing sugar on acid hydrolysis, and optical rotation to the glycogen from other animal tissues, accounts for about one-half of the total carbohydrate of unfertilized *Arbacia* eggs.

It has also been found that, during the first few hours of development while cell division was actively proceeding, the fraction of the total carbohydrate which was oxidized or converted to lactic acid was very small. Indeed, with certain samples of eggs, there appeared to be, within the limit of accuracy of the experimental method, no carbohydrate consumption during the first 3 to 6 hours of development. Under these conditions a substantial proportion of the



oxygen consumption could be provisionally accounted for, on the basis of measurements of concurrent ammonia production, by oxidation of protein.

### *Experimental Methods*

Mature *Arbacia* eggs were obtained at Woods Hole during June, July, and August, 1941. They were shed, washed, and, where necessary, fertilized as described in previous papers of this series.

*Analysis for Total Carbohydrate.*—The eggs were prepared for carbohydrate analysis, and the wet weight and nitrogen content determined either by total nitrogen analyses or by calculation from hematocrit values as described below in the section on egg number-volume-weight-nitrogen content-conversion factors. To find the total carbohydrate, the eggs were packed by centrifuging in a 15 cc. tube, taken up in 1 N HCl, and hydrolyzed for 1 hour in a boiling water bath. Confirming Perlzweig and Barron, it was observed that longer hydrolysis yielded no more reducing material. The proteins and protein cleavage products were precipitated by adding an equal volume of a solution 0.2 M in mercuric acetate, and 0.2 M in HCl; the excess mercury was removed with H<sub>2</sub>S, and the H<sub>2</sub>S removed with an air stream. An aliquot of the solution was then neutralized, made to a known volume, and filtered to remove echinochrome.<sup>1</sup> The reducing material was then determined either by the Folin-Wu (4) copper method or by the Folin (5) ferricyanide method. When both methods were applied to the same sample, they always yielded the same values for egg-reducing material (see Table II). Permutit and Lloyd's reagent were used in an effort to detect and remove any non-specific reducing materials, but the reducing values obtained after such treatment were the same as before.

*Determination of Glycogen.*—Glycogen was isolated from the unfertilized *Arbacia* eggs by the following version of Pflüger's (6) method: The packed eggs were mixed with 1 volume of 60 per cent NaOH solution and hydrolyzed for 3 hours in a boiling water bath. Longer hydrolyses up to 6 hours did not change the glycogen values obtained from a given egg sample. The mixture was then diluted with an equal volume of water, and alcohol added to give a final concentration of 66 per cent by volume. The glycogen was allowed to precipitate in the ice box, usually overnight, centrifuged down, and dried. For volumetric analysis, the glycogen was redissolved in water and aliquots were subjected to hydrolysis and sugar analysis as described above. For polarization analysis, the glycogen samples were reprecipitated several times with alcohol and partially decolorized with charcoal. For the comparison analyses of Table V the samples for volumetric analyses were withdrawn, after the readings, directly from the polarimeter tube.

Following the suggestion of Blanchard (3) a number of egg samples were extracted repeatedly with a 50-50 mixture of ethyl alcohol and ethyl ether, then dried, before the alkaline hydrolysis. This procedure, which was employed to remove lipoidal

<sup>1</sup> Further investigation of the conditions for echinochrome removal showed that in order to obtain optimum precipitation, the solution had to be at least 0.75 M in NaCl (or its equivalent in ionic strength) and at a pH above 6; the presence of divalent ions, such as calcium or magnesium, also favored precipitation of the echinochrome.

materials which might interfere with subsequent precipitation of the glycogen from alcohol, caused a loss of approximately 25 per cent of the total carbohydrate of the egg, and did not improve the glycogen yields or the purity of the isolated glycogen.

The optical rotations were measured in a 2 dm. tube on a polarimeter reading to  $\pm 0.05^\circ$ , using the most concentrated solution of glycogen which would transmit sufficient light to permit matching of the fields of the polarimeter. Ten observations by each of two operators were made on each solution. The maximum variation of readings by individual observers was  $\pm 0.1^\circ$ .

*Procedure for Metabolic Determinations.*—The eggs were obtained, washed, and fertilized in a large volume of sea water, 95 to 100 per cent fertilization being obtained in each case. The fertilized eggs were allowed to settle, the supernatant sea water removed, enough 0.11 M pH 8.0 glycylglycine buffer (330 mg. glycylglycine plus 1.2 cc. M NaOH to 25 cc. with sea water) added to give a final concentration of 0.01 M, and the volume adjusted to give the desired per cent of eggs by volume. For experiments with normal eggs, the main compartment of each Warburg flask received 2.0 cc. of a 3.0 per cent egg suspension. When 4,6-dinitro-*o*-cresol was used, each flask received 2 cc. of a 1.5 per cent egg suspension plus 0.1 cc. of a solution of 4,6-dinitro-*o*-cresol in sea water. The center cup received 0.5 cc. of 2.5 N HCl, and the side arm 0.5 cc. of freshly filtered, saturated, barium hydroxide solution. The flasks were placed in the bath at 30 minutes after fertilization and equilibrated, at 20° C., with air as gas phase, for 20 to 30 minutes, a number of preliminary manometric readings being taken during this time. The initial reading for the actual determination was taken at 50 or 60 minutes after fertilization. Each determination was made in duplicate, the first pair of flasks having the contents of the main vessel, center cup, and side arm mixed immediately following the initial reading, with subsequent pairs being similarly treated at the times shown in the tables. The flasks were shaken at 60 cycles with a 4 cm. amplitude. Under these conditions, the eggs were just at the point of first division at 1 hour after fertilization, the time at which measurements began, developed to swimming blastulae at 10 hours, gastrulae at 15 hours, and short-armed plutei at 24 hours. The shaking rate was shown to be adequate for maintenance of oxygen equilibrium and the average oxygen consumption for every sample of eggs fell very close to the average value of 3.1 c.mm. per 10 c.mm. eggs per hour as observed in this laboratory for several years.

At the end of the experiment, the total contents of each duplicate pair of Warburg flasks were carefully rinsed into a 10 cc. volumetric flask and made to volume. Suitable aliquots from each sample were then analyzed for sugar by the Folin ferricyanide method as described above, for lactic acid by the colorimetric method of Barker and Summerson (7), and for ammonia by nesslerization of the distillate obtained after 1 hour aeration of alkalinized samples by the technique of Folin (8).

#### EXPERIMENTAL RESULTS

*Nitrogen Content in Relation to Number, Volume, Wet Weight, and Dry Weight of Arbacia Eggs.*—For comparison of the present data with that presented on various phases of *Arbacia* egg metabolism in previous papers of this series and by other investigators, it was desirable to know the nitrogen content of the eggs under the present conditions of experiment. For this purpose, unfertilized

*Arbacia* eggs were, as in the actual experiments, freed of ovarian material by sifting through two layers of cheese cloth, then washed by allowing them to settle through two changes of sea water. The percentage of eggs by volume in the suspension used in any experiment was measured by the hematocrit method previously described (9). An aliquot of the egg suspension was removed for total nitrogen analyses, digested, and nesslerized by the micro Kjeldahl method of Folin (10), the color being measured photoelectrically at 400 m $\mu$ . Another aliquot from the same suspension was packed by centrifuging for 5 minutes at 2000 gravity in a tared 15 cc. centrifuge tube terminating in a 5 mm. capillary. The supernatant sea water was carefully drawn off by suction, the last portions of extraneous sea water being removed from the inside of the tube with filter paper. The tubes were weighed with contents and then dried to constant weight at 110°C.

The results of four experiments are given in Table I. By the present method, the eggs were found to contain 23.9 per cent of the measured wet weight as solids, or 25.9 per cent of the wet weight calculated from the egg volume and a density of 1.09 as solids. In 1909 McClendon<sup>2</sup> (11) reported the solid content of unfertilized eggs to be 22.6 per cent. In 1940 Ballentine (12) reported 265 mg. dry weight per cc. eggs (volume determined by diameter measurements on individual eggs in conjunction with egg counts); on the assumption of an egg density of 1.09 (13), this gives 24.2 per cent solids.

Ballentine also reported 0.107 mg. nitrogen per mg. dry weight, which is essential agreement with the value of 0.101 mg. nitrogen per mg. dry weight found in the present experiments. If the average value of 95,000 eggs per cc. of 2 per cent eggs, used in this laboratory for several years (9), is converted on the basis of the data of Table I a figure of 5.93 mg. nitrogen per 10<sup>6</sup> cells is obtained; Ballentine found 5.86 mg. nitrogen per 10<sup>6</sup> cells. Since the data of Table I appear, therefore, to be in satisfactory agreement with previous data, the factors for nitrogen per unit of egg volume or per number of cells being particularly reproducible, no further experiments of this type were made.

On the basis of Ballentine's data and that of Table I, unfertilized *Arbacia* eggs contain approximately 63 to 67 per cent of the dry weight as protein.

*Total Carbohydrate Content of Arbacia Eggs.*—The results of twenty-one determinations of the total carbohydrate content of *Arbacia* eggs are given in Table II. The average figure was 110 mg. carbohydrate (calculated as glucose) per gm. egg protein.

It will be noted that the values obtained during the early part of the season tend to be lower than those obtained later. This may be in part connected

<sup>2</sup> In a previous paper of this series the solid content of *Arbacia* eggs was taken to be 18.1 per cent, as quoted from McClendon by Harvey (see Harvey review (3)). This is the solid content calculated from data on various egg fractions obtained by centrifuging. The figure given by McClendon for the solid content of the whole egg was 22.6 per cent.

TABLE I

*Total Nitrogen and Solid Content of Unfertilized Eggs of Arbacia punctulata*

Date	Egg volume by hematocrit	Egg wet weight		Egg dry weight	Total egg nitrogen	Per cent dry weight		Nitrogen per cc. of eggs	Nitrogen per mg. egg dry weight
		Calculated from density of 1.09 (12)	Found			Calculated	Found		
	cc.	mg.	mg.	mg.	mg.			mg.	mg.
7-3-41	0.272	296	323	76	7.7	25.6	23.6	28.2	0.101
7-3-41	0.435	473	522	128	12.1	27.0	24.5	27.8	0.095
7-3-41	0.465	505	575	142	13.1	28.2	24.7	28.1	0.092
7-3-41	0.370	402	406	92	10.5	22.9	22.7	28.4	0.114
Mean.....						25.9	23.9	28.1	0.101

TABLE II

*Total Acid-Hydrolyzable Carbohydrate Content (As Glucose) of Eggs of Arbacia punctulata*

Date	Eggs unfertilized (U) or at 1 hr. after fertilization (F) at 20° C.	Total nitrogen of egg sample (N)	Protein in egg sample (N × 6.25)	Total glucose in egg sample		Glucose per gm. of egg protein†
				By ferricyanide method	By copper method	
		mg.	mg.	mg.	mg.	mg.
6-21-41	U	0.51	3.19	0.243		76
6-24-41	U	1.39	8.69	0.814		94
6-26-41	U	0.47	2.93	0.229		82
7-9-41	U	0.95	5.94	0.597	0.603	101
					0.613*	
7-9-41	U	1.38	8.62	0.873	0.855	98
					0.857*	
7-9-41	U	1.32	8.25	0.925	0.917	99
					0.937*	
7-9-41	U	1.61	10.1	1.20	1.17	119
					1.19*	
7-16-41	U	5.17	32.3	3.62	3.56	111
7-24-41	U	7.07	44.2	4.13		94
7-30-41	F	1.69	10.6	1.01		96
8-2-41	F	0.85	5.3	0.78		147
8-21-41	F	1.69	10.6	1.53		145
8-23-41	F	1.69	10.6	1.53		143
8-24-41	F	1.53	9.6	0.91		96
8-25-41	F	1.69	10.6	1.48		140
8-27-41	F	1.69	10.6	1.48		140
8-28-41	F	1.12	7.0	1.44		136
8-29-41	U	2.24	14.0	1.58		113
8-29-41	U	2.26	14.1	1.80		128
8-29-41	U	4.11	25.7	3.21		125
8-29-41	U	5.26	32.9	4.02		122
Mean.....						110

\* After treatment with Lloyd's reagent.

† Average of all determinations on the sample.

with the fact that eggs used after August 1st came, in the majority of cases, from urchins which had been stored in aquaria inside the laboratory for periods of 2 to 4 weeks; the eggs used in the early experiments came from urchins stored for only a few days in the laboratory.

No reason can be given for the fact that the present carbohydrate values are twice as high as those reported by Perlzweig and Barron, especially since the methods for hydrolyzing and determining carbohydrate, removal of interfering substances, and determination of egg nitrogen content were essentially the same in the two investigations. In view of the variation among the separate determinations of Table II, the discrepancy may possibly be attributable to variations in the source and handling of the urchins.

The egg hydrolysates, after removal of interfering substances by the mercuric acetate precipitation, did not reduce neutral or acid ferricyanide solutions. Also, confirming Perlzweig and Barron (2) and Blanchard (3), typical glucosazone crystals appeared in concentrated hot egg hydrolysates at the same rate as in known glucose solutions of the same reducing power, and melted simultaneously with known glucosazone crystals at 204°C. (uncorrected). However, not all of the total carbohydrate was glucose, since the presence of some pentose was shown by formation of furfural on boiling the isolated sugar with concentrated HCl.

It was of interest to determine how much of the reducing carbohydrate might arise from the jelly surrounding the eggs. To investigate this question the following experiment was conducted.

A suspension of eggs was divided into two parts. One part was analyzed in the usual way, giving 1.15 mg. glucose and 14.0 mg. protein; *i. e.*, 82 mg. of glucose per gm. of protein. The eggs in the other part, after packing by centrifugation and removal of the supernatant sea water, were shaken gently in isotonic NaCl (0.55 M), packed, and the supernatant NaCl solution decanted. The eggs were washed again with isotonic NaCl and the two NaCl washings combined. The eggs, freed of jelly, were then analyzed as before, giving 1.32 mg. glucose and 14.7 mg. protein; *i. e.*, 90 mg. of glucose per gm. of protein. The NaCl solution containing the jelly was evaporated to 25 cc. This solution contained no demonstrable reducing material, but was found to contain the nitrogen equivalent of 1.56 mg. protein. Adding the nitrogen found in the jelly to that found in the eggs from which it was removed gave a total of 16.3 mg. protein which, divided into the glucose found in the eggs, gave 81 mg. of glucose per gm. protein.

This experiment was repeated with similar results. It seems clear that the jelly, though accounting for about 10 per cent of the nitrogen of the eggs, contains only an inappreciable fraction of the hydrolyzable reducing sugar of the eggs; thus, the reducing carbohydrate is in the egg where it is a potential source of energy.

*Glycogen Content of Arbacia Eggs.*—The results of a number of glycogen de-

terminations on unfertilized *Arbacia* eggs are given in Table III. The glycogen content here observed (40 to 57 mg. per gm. egg protein) is of the same order as that (50 to 80 mg. per gm. egg protein) obtained in the unpublished experiments of Blanchard (see (3)). It is also of the same order as that found in the eggs of the European sea urchins *Paracentrotus lividus* and *Echinus esculentus* (see Örström and Lindberg (14) for a summary of the total carbohydrate and glycogen contents of these eggs).

Since only about one-half of the total hydrolyzable carbohydrate was accounted for as glycogen, recovery of a known amount of glycogen was attempted.

2 aliquots of eggs from the same batch were carried through the procedure for glycogen isolation. The first sample, containing 4.03 gm. wet eggs, gave 66 mg. total

TABLE III  
*Glycogen Content of Unfertilized Arbacia Eggs*

Date	Total acid-hydrolyzable carbohydrate (as glucose) per gm. of egg protein	Glycogen (as glucose) per gm. of egg protein	Ratio of glycogen to total acid-hydrolyzable carbohydrate
	mg.	mg.	per cent
6-24-41	94	44	47
7-16-41	109	55	51
7-24-41	94	49	52
7-24-41	109	57	52
8-29-41	113	5	45
8-29-41	128	57	44
8-29-41	125	40	32
8-29-41	122	50	41
Mean.....			46

glucose and 34.4 mg. glycogen (calculated as glucose); i. e., 52 per cent of total hydrolyzable carbohydrate as glycogen. To the second sample, containing 2.02 gm. wet eggs, glycogen (Pfanstiehl) equivalent to 31.3 mg. glucose (as shown by analysis) was added. On analysis, this second sample gave 47.5 mg. glycogen (calculated as glucose), of which 17.2 mg. ( $0.5 \times 66 \times 0.52$ ) came from the eggs and 30.3 mg. came from the 31.3 mg. added, indicating a recovery of 97 per cent.

Further experiment (Table IV) indicated that the principal loss of carbohydrate occurred at the ether-alcohol extraction and the alcohol precipitation steps of the glycogen isolation, only a negligible loss being incurred during the alkaline hydrolysis.

The glycogen isolated from unfertilized *Arbacia* eggs by the present procedure gave the expected red-brown color test with iodine solutions. Further evidence for the identity of *Arbacia* glycogen with that in other animal tissues was pro-

vided by experiments in which glycogen, after several alcohol precipitations, was estimated from its optical rotation before acid hydrolysis and also by the ferricyanide method after acid hydrolysis. Results of six experiments (Table V) show the average value obtained by hydrolysis to be 98 per cent of that obtained by rotation.

TABLE IV

*Loss of Hydrolyzable Carbohydrate at Various Stages in the Isolation of Glycogen from Unfertilized Arbacia Eggs*

	Total acid-hydrolyzable carbohydrate (as glucose) per gm. of egg protein	Cumulative loss of original glucose
	mg.	per cent
Original eggs.....	109	0
Following drying with 50 per cent alcohol-50 per cent ether.....	82	25
Following alkaline hydrolysis.....	80	27
Following precipitation with 66 per cent alcohol.....	55	50

TABLE V

*Comparison of Glycogen Analyses by Measurement of Optical Rotation before Acid Hydrolysis with Those by the Ferricyanide Method after Acid Hydrolysis*

Rotation*	Glycogen per cc. (calculated as glucose)		Ratio of glucose by ferri- cyanide method to glucose by rotation method
	By rotation method	By ferricyanide method	
degrees	mg.	mg.	per cent
0.7	1.8	2.0	111
1.1	2.8	2.5	89
1.9	4.8	4.4	92
5.1	13.0	12.0	92
1.6	3.1	3.0	97
4.5	11.5	12.2	106
Mean.....			98

\* Larger rotations could not be read because of the traces of color which could not be removed from the egg glycogen. The specific rotation of glycogen is 196.6° (15).

*Metabolic Determinations.*—Having established that *Arbacia* eggs contain a considerable amount of carbohydrate in a form presumably subject to use as a metabolic substrate, it was of interest to make a preliminary survey of the actual carbohydrate consumption of the eggs as they developed under the conditions of experiment used in previous papers of this series. Measurements of carbohydrate consumption and of formation of various metabolic products

were made at intervals between 1 and 25 hours<sup>3</sup> after fertilization at 20°C. (Tables VI and VII.)

The results indicate that during the first 3 to 6 hours of development, the period in which cell division is most actively proceeding, the fertilized eggs may or may not consume significant amounts of carbohydrate. Indeed, in two experiments (Nos. 89 and 91), there appeared to be little or no carbohydrate consumed in the 6 hour period between the 1st and the 7th hour of development. Since the experimental error in the glucose determinations was about  $\pm 2$  per cent, complete absence of carbohydrate consumption in these cases was not established, but the maximum carbohydrate consumption allowed by the error of the determination (at most  $0.3 \times 10^{-6}$  moles glucose per gm. wet eggs) could not account for more than 15 per cent at most of the oxygen consumption during the first 6 hours. From the 15th to the 24th hour, however, considerable amounts of carbohydrate were consumed by every egg sample.

Of the carbohydrate consumed in any given instance, a negligible proportion was converted to lactic acid during the period from the 1st to the 24th hour of development. It is true, as pointed out by previous investigators (2, 3), that the eggs contain some lactic acid shortly after fertilization, but neither production nor consumption of lactic acid appeared to be of quantitative importance as energy-yielding processes for *Arbacia* eggs during their first 24 hours of development.

In every experiment, *Arbacia* eggs were found to produce substantial amounts of ammonia. In a number of cases, the oxygen which would have been consumed in producing the observed amount of ammonia from complete oxidation of protein (see Dickens (16)) agreed with that actually consumed. This finding suggested that the eggs might derive a considerable fraction or, in the absence of carbohydrate consumption, all of the energy for early development from protein oxidation.

Unfortunately, the situation is not so simple as this, since the oxygen consumption required for protein oxidation was, in a number of experiments,

<sup>3</sup> The question of the degree to which bacterial growth may contribute to the metabolic values obtained at the 15th and 24th hours cannot be definitely answered, but it may be noted that the oxygen consumption of the various egg samples remains fairly constant from experiment to experiment, giving no reason to suspect chance bacterial growth. In control experiments, an attempt was made to detect and prevent any potential bacterial growth by use of a series of closely spaced concentrations of sodium ethylmercurithiosalicylate. As the concentration of this substance was increased, there was no effect on the respiration of the egg suspension until a concentration of  $5 \times 10^{-6}$  M was reached. Beyond this concentration, the respiration of the eggs was inhibited along a smooth concentration effect curve which gave no indication that any organism except the eggs was contributing, during a 24 hour period, to the respiration of the egg suspension.



TABLE VI

*Oxygen Consumption, Carbohydrate Content, Lactic Acid Content, and Ammonia Nitrogen Content of Arbacia Eggs from the 1st to the 25th Hour of Development at 20° C.*

Exp. No.	Date	Wet weight of eggs used	Time after fertilization	Total oxygen consumed	Total egg carbohydrate content (as glucose)	Total egg lactic acid	Total egg ammonia nitrogen
		mg.	hrs.	c. mm.	μg.	μg.	μg.
45	7-30-41	66	1		1012	No analyses	20
			4	51	963		34
			7	110	925		45
			11	222	835		54
			16	352	695		63
			24	563	493		71
89	8-21-41	66	1		1530	16	20
			4	52	1490	14	31
			7	124	1530	15	36
			11	221	1190	19	40
			16	463	1060	19	44
			25	808	985	16	71
90	8-23-41	66	1		1505	7	22
			4	56	1505	7	38
			7	134	1475	7	40
			10	232	1475	5	40
			16	413	1440	6	58
			25	877	1135	9	75
91	8-24-41	60	1		910	9	16
			4	45	900	6	7
			7	117	910	6	5
			11	242	905	7	40
			16	364	910	7	57
			25	615	683	8	94
92	8-25-41	66	1		1480	8	40
			4	61	1390	8	55
			7	152	1400	4	55
			11	286	1230	5	83
			16	488	1260	6	91
			25	890	1080	7	107
96	8-27-41	66	1		1479	15	29
			4	75	1470	6	33
			7	173	1458	6	39
			10	326	1375	7	59
			16	425	1310	7	55
			25	798	1023	8	112

TABLE VI—*Concluded*

Exp. No.	Date	Wet weight of eggs used	Time after fertilization	Total oxygen consumed	Total egg carbohydrate content (as glucose)	Total egg lactic acid	Total egg ammonia nitrogen
		mg.	hrs.	c. mm.	μg.	μg.	μg.
46*	8-2-41	33	1		775	10	13
			4	52	753	10	20
			7	111	722	11	25
			10	164	657	11	23
			16	265	620	14	54
			26	438	548	10	113
99A†	8-28-41	44	1		1438	5	8
			11	177	1058	6	55
B			1		1330	5	21
			11	330	1330	4	53
C			1		1250	6	20
			11	136	1250	6	25

\* All samples contained  $1 \times 10^{-6}$  M 4,6-dinitro-*o*-cresol.

† The A samples were normal eggs; the B and C samples contained  $8 \times 10^{-6}$  M and  $6.4 \times 10^{-6}$  M 4,6-dinitro-*o*-cresol, respectively.

either greater or less than the oxygen consumption which was not accounted for by carbohydrate oxidation. For the periods in which the total oxygen consumption could not be accounted for by the sum of carbohydrate and protein oxidation (see particularly experiment 96, 1 to 7 hours), the oxidation of other substrates, *e.g.* fat, possibly accounts for the remaining oxygen consumed. In this connection, it is suggestive that the respiratory quotients for such periods were low.

Clearly, much further work is required to establish the relative importance of various metabolic substrates in *Arbacia* egg development. For the present, the important point is that the *Arbacia* eggs in the separate samples consumed oxygen at essentially the same rate, and developed normally at the same rate irrespective of whether they used carbohydrate- or ammonia-yielding substances as their principal sources of foodstuff. This suggests that if the energy for cell division of *Arbacia* eggs comes from a particular sequence of respiratory processes, this must be one which is common to both carbohydrate oxidation and protein oxidation, or one which is concerned with the utilization of some other substance not accounted for here.

In previous experiments (9) it has been shown that 4,6-dinitro-*o*-cresol, when used in optimum concentration, can raise the oxygen consumption of fertilized *Arbacia* eggs to 3 or 4 times the normal level over a period of 3 hours. The present experiments show (Table VIII) that  $8 \times 10^{-6}$  M or  $10^{-5}$  M 4,6-dinitro-*o*-cresol, in raising the oxygen consumption by almost 100 per cent over a more

TABLE VII

*Relation of Oxygen Consumption to Carbohydrate Consumption and Ammonia Production in Normal Fertilized Arbacia Eggs at 20°C., As Calculated for 1 Gm. Wet Eggs, from the Data of Table VI*

1	2	3	4	5	6	7	8	9
Exp. No.	Period of measurement	Oxygen consumed	R. Q.	Glucose used	Oxygen equivalent of glucose used	Ammonia nitrogen (NH <sub>3</sub> -N) formed	Protein oxygen equivalent of ammonia nitrogen	Sum of columns 6 and 8
	hrs. after fertilisation	moles $\times 10^6$		moles $\times 10^6$	moles $\times 10^6$	moles $\times 10^6$	moles $\times 10^6$	moles $\times 10^6$
45	1- 4	3.45	0.94	0.41	2.46	1.51	6.63	9.09
	1- 7	7.44	1.00	0.73	4.37	2.70	11.9	16.3
	1-11	15.0	0.92	1.49	8.93	3.68	16.2	25.1
	1-16	23.8	0.97	2.66	15.9	4.65	20.4	36.3
	1-24	38.1	0.92	4.36	26.2	5.51	24.3	50.5
89	1- 4	3.51	0.87	0.34	2.04	1.19	5.23	7.27
	1- 6	8.38	0.87	0	0	1.73	7.60	7.60
	1-11	14.9	1.04	2.86	17.1	2.16	9.50	26.6
	1-16	31.4	0.82	3.96	23.8	2.60	11.4	35.2
	1-25	54.7		4.59	27.5	5.53	24.4	51.9
90	1- 4	3.79	0.76	0	0	1.73	7.60	7.60
	1- 7	9.07	0.82	0.25	1.50	1.95	8.58	10.1
	1-10	15.7	0.84	0.25	1.50	1.95	8.58	10.1
	1-16	28.0	0.87	0.55	3.24	3.89	17.1	20.3
	1-25	59.4		3.10	18.8	5.74	25.2	44.8
91	1- 4	3.35	0.78	0.09	0.54	-1.07	-4.71	
	1- 7	8.71	0.95	0	0	-1.31	-5.77	
	1-11	18.0	0.90	0.04	0.24	2.86	12.6	12.8
	1-16	27.2	0.94	0	0	4.88	21.5	21.5
	1-25	45.8	0.98	2.10	12.6	9.30	40.9	53.5
92	1- 4	4.13	0.95	0.76	4.56	1.63	7.18	11.7
	1- 7	10.3	0.90	0.67	4.02	1.63	7.18	11.2
	1-11	19.3	0.93	2.10	12.6	4.66	20.6	33.2
	1-16	33.0	0.86	1.85	11.1	5.53	24.4	35.5
	1-25	60.2	0.80	3.36	20.2	7.26	32.0	52.2
96	1- 4	5.07	0.82	0.08	0.48	0.43	1.90	2.38
	1- 7	11.7	0.75	0.18	1.08	1.08	4.75	5.83
	1-11	22.0	0.75	0.88	5.27	3.25	14.3	19.6
	1-14	28.8	0.79	1.42	8.51	2.82	12.4	20.9
	1-25	54.0	0.84	3.74	22.4	8.98	39.6	62.0

extended period, does not induce the consumption of sufficient excess carbohydrate to account for the excess oxygen consumed.

This effect is especially striking in experiment 99. While the oxygen consumption was raised by  $8 \times 10^{-6}$  M 4,6-dinitro-*o*-cresol from 18.0 to  $33.6 \times 10^{-6}$  moles per gm. wet eggs, the carbohydrate consumption from the 1st to the 11th hour was reduced, within the limits of experimental error, to zero. However, the low carbohydrate content of the 4,6-dinitro-*o*-cresol treated eggs at 1 hour indicated that there had been, during the 30 minute equilibration period, a considerable consumption of carbohydrate. The ammonia production from the 1st to the 11th hour was also reduced to a point where the total oxygen

TABLE VIII

*Relation of Oxygen Consumption to Carbohydrate Consumption and Ammonia Production in 4,6-Dinitro-*o*-Cresol Treated-Fertilized Arbacia Eggs, as Calculated for 1 Gm. Wet Eggs from the Data of Table VI*

1	2	3	4	5	6	7	8	9	10
Exp. No.	Period of measurement	Concentration 4,6-dinitro- <i>o</i> -cresol	Oxygen consumed	R. Q.	Glucose used	Oxygen equivalent of glucose used	Ammonia nitrogen (NH <sub>3</sub> -N) formed	Protein oxygen equivalent of ammonia nitrogen (NH <sub>3</sub> -N $\times$ 4.4)	Sum of columns 7 and 9
	hrs. after fertilization	moles per l. $\times 10^6$	moles $\times 10^6$		moles $\times 10^6$	moles $\times 10^6$	moles $\times 10^6$	moles $\times 10^6$	moles $\times 10^6$
99A	1-11	0	18.0	0.93	4.81	28.8	7.63	33.6	62.4
B	1-11	8	33.6	0.89	0	0	5.19	22.8	22.8
C	1-11	64	13.8	0.92	0	0	0.81	3.57	3.57
46	1-4	10	7.04	0.97	0.37	2.22	1.52	6.70	8.92
	1-7	10	15.0	0.89	0.89	5.33	2.60	11.4	16.7
	1-10	10	22.2	0.87	1.99	11.9	2.16	9.51	21.4
	1-16	10	35.8	0.91	2.61	15.6	8.88	39.1	54.7
	1-26	10	59.3	0.91	3.82	22.9	21.6	95.1	118.0

consumption could not be accounted for by oxidation of protein. At  $6.4 \times 10^{-6}$  M 4,6-dinitro-*o*-cresol, a concentration greater than the optimum for respiratory stimulation, the carbohydrate consumption was also reduced to zero and the proportion of oxygen consumption accounted for by protein oxidation was even smaller than at  $8 \times 10^{-6}$  M. Further experiments to elucidate this effect and its relation to the cell division-blocking action of this class of compounds are obviously desirable.

## SUMMARY

1. Under the present conditions of experiment, *Arbacia* eggs were found to contain an average of 110 mg. of acid-hydrolyzable carbohydrate (calculated

as glucose) per gm. of egg protein. This carbohydrate was almost all in the egg proper, little or none being found in the jelly.

To permit conversion of the data to other bases of reference the relation of nitrogen content to wet and dry weight and to egg number were determined. The eggs were found to contain 23.9 per cent solids, 0.10 mg. nitrogen per mg. dry weight, and 5.93 mg. nitrogen per  $10^6$  cells. From these results, about 7 per cent of the egg dry weight is acid-hydrolyzable carbohydrate and about 65 per cent is protein.

2. Approximately one-half of the total acid-hydrolyzable carbohydrate was isolated in the form of an alkali-stable, alcohol-precipitable carbohydrate. This substance gave a typical glycogen color test with iodine, yielded glucose on acid hydrolysis, and had, within the limits of experimental error, the same optical rotation as glycogen from other animal sources. Since known amounts of glycogen were completely recovered when carried through the isolation process, the nature of one-half of the acid-hydrolyzable carbohydrate of *Arbacia* eggs remains undetermined.

3. In order to gain some estimate of the extent to which *Arbacia* eggs utilize their total carbohydrate for development, determinations of the oxygen consumption, respiratory quotient, carbohydrate consumption, lactic acid production, and ammonia production were made. While all samples of eggs were found to utilize carbohydrate from the 15th to the 24th hours of development at 20°C., certain samples of eggs consumed little or no carbohydrate from the 1st to the 6th hours, the period during which cell division proceeds most rapidly. In a number of instances where carbohydrate breakdown was lacking, a substantial proportion of the oxygen consumption could be accounted for on the basis of processes involving oxidation of protein or protein breakdown products.

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## STUDIES ON CELL METABOLISM AND CELL DIVISION

### VII. OBSERVATIONS ON THE AMOUNT AND POSSIBLE FUNCTION OF DIPHOSPHOTHIAMINE (COCARBOXYLASE) IN EGGS OF *ARBACIA PUNCTULATA*

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The experiments here reported were made as part of an experimental inquiry into the mechanism of energy transfer for cell division in the fertilized eggs of *Arbacia punctulata*. It has of late become increasingly apparent that efforts to relate metabolism to function must be based on a detailed knowledge of the metabolic processes in the particular tissue in question. Accordingly, for some time an effort has been made in this laboratory to establish for *Arbacia* eggs (a) the concentration of certain catalytic substances which are known to be important for respiratory electron transfer, and (b) the relation of these agents to the individual metabolites and their activating proteins. In the course of this program, it has been shown (1) that the eggs contain an enzyme which, with cytochrome c as a substrate, displays the properties of an electromotively active iron-porphyrin oxidative catalyst. It has also been shown that *Arbacia* eggs contain flavin-adenine dinucleotide (2).

The present paper deals with the occurrence of diphosphothiamine (cocarboxylase) in *Arbacia* eggs. In certain cells, this substance is involved in at least one type of oxidative energy transfer, namely, the coupling of pyruvate oxidation with phosphorylation (3-6); diphosphothiamine also catalyzes a number of other metabolic processes in animal tissues, especially those concerned with both aerobic and anaerobic utilization of pyruvic acid (7). It was therefore of interest to find that the diphosphothiamine content of *Arbacia* eggs, 2-3 micrograms per gram wet weight, is comparable with that in other animal tissues (8, 9), and that crude enzyme preparations from *Arbacia* eggs can cause pyruvate to disappear both aerobically and anaerobically.

#### *Experimental Methods and Materials*

**Principle of Diphosphothiamine and Pyruvate Determinations.**—Diphosphothiamine was estimated by manometric measurement of the rate of decarboxylation of pyruvic acid under conditions where the rate of CO<sub>2</sub> liberation was proportional to the concentration of diphosphothiamine.

Pyruvic acid was estimated, as suggested by Warburg and Christian (10), by manometric measurement of the total CO<sub>2</sub> liberated by carboxylase from solutions of pyruvic acid.



*Preparation and Test of Carboxylase.*—Carboxylase was prepared from dried top ale yeast,<sup>1</sup> essentially according to the directions of Green, Herbert, and Subrahmanyam (11). Both the preparation and splitting of the carboxylase were based on the methods of Benditt (12); we are greatly indebted to Dr. Benditt for putting his results at our disposal. As here employed, the carboxylase preparation was as follows: 150 cc. of 0.067 M phosphate buffer, pH 7.2, were warmed to 40° C. in a 500 cc. Erlenmeyer flask, and 50 gm. of dried yeast were added slowly with shaking. The mixture was kept at 38° C. for 1 hour, with occasional shaking; 50 cc. water were then added. The mixture was centrifuged for 20 minutes in an angle head centrifuge at about 2000 gravity. The residue was discarded. To the supernatant liquid were added 14 cc. 0.5 M phosphate buffer, pH 7.2, followed by 7 cc. of 1 M calcium acetate drop by drop with constant stirring. The mixture was again centrifuged for 10 minutes in an angle head centrifuge at about 2000 gravity. The precipitate was discarded. 38 gm. reagent grade ammonium sulfate were added slowly to each 100 cc. of the supernatant liquid; the suspension was stirred until the salt was in solution. The carboxylase was used in the form of this suspension and could be preserved for several weeks at 8° C. without loss of activity.

The activity of carboxylase was determined manometrically in Warburg vessels as described below under test system for diphosphothiamine in *Arbacia* eggs. Results were expressed in terms of units (11) of activity per cubic centimeter; *i. e.*, the reciprocal of the volume of enzyme suspension necessary to produce 100 c.mm. CO<sub>2</sub> in 3 minutes at 30° C. For the test, the main compartment of each vessel contained: 0.05 cc. carboxylase suspension; 0.05 cc. of a solution containing 1 mg. synthetic diphosphothiamine per cc.; 0.35 cc. 0.25 M phosphate buffer, pH 6.4; 2.35 cc. water. The side arm contained 0.5 cc. 1.6 M sodium pyruvate.

*Preparation and Test of Split Carboxylase.*—To a mixture of 20 cc. carboxylase suspension, prepared as described above, and 40 cc. water in a 250 cc. graduate, 8 cc. concentrated ammonia were added; the mixture was allowed to stand 10 minutes at room temperature. Then 72 cc. of a saturated solution of reagent grade ammonium sulfate were added and thoroughly mixed with the ammoniacal solution. The resulting suspension was centrifuged in an angle head centrifuge for 30 minutes and the supernatant discarded. The precipitate was taken up in 10 cc. 0.25 M phosphate buffer, pH 6.4, the solution transferred to a 25 cc. graduate, and the centrifuge tubes rinsed with 5 cc. of the buffer which was then added to the enzyme solution. To the combined solutions 9.5 gm. reagent grade ammonium sulfate were added; the mixture was stirred until the salt dissolved. After adjustment to pH 6.4 the volume was made up to 25 cc. with the 0.25 M phosphate buffer. This suspension of carboxylase protein, which was virtually free from diphosphothiamine, was used for

<sup>1</sup> Top brewer's yeast; obtained from the Croft Brewery, Boston, Massachusetts, was collected on large Buchner funnels and washed with 5 volumes of tap water, followed by 1 to 2 volumes of distilled water. Filtration by suction was continued until the cake started to break up. The yeast was spread on sheets of filter paper, in as fine a state of division as possible, and dried overnight under a fan at room temperature. The particles were crushed by rolling with a large bottle and sifted through a fine screen. The quicker the drying process, the more active the dried yeast.

the diphosphothiamine determinations; it could be preserved for a week or two at 8° C.

For the test, the main compartment of each Warburg vessel received: 0.05 cc. of split carboxylase; 0.3 cc. of a solution containing 410 mg.  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  per 100 cc. in water; 0.05 cc. of a solution containing 1 mg. synthetic diphosphothiamine per cc.; 0.35 cc. 0.25 M phosphate buffer, pH 6.4; 2.05 cc. water. The side arm contained 0.5 cc. 1.6 M sodium pyruvate. The test was run, and the results expressed, as described above for the complete carboxylase enzyme.

By this method of preparation, solutions containing 5 to 8 units of carboxylase per cc. (when resaturated with diphosphothiamine) were obtained.

This method of preparing split carboxylase yielded enzyme preparations which gave, in the above test, a blank value (*i. e.* in absence of added diphosphothiamine) equal to only 0 to 5 per cent of the value obtained when the enzyme was saturated with added diphosphothiamine. Any blank value initially displayed by the split carboxylase tended to decrease or disappear after a day or two of storage at 8° C.

*Preparation and Test of Sodium Pyruvate.*—17.6 gm. Eastman Kodak Co. pyruvic acid (No. 498) were cooled in an ice-salt bath and neutralized drop by drop with strong (5 to 10 N) NaOH, the temperature being kept below 2° C. As the end point was approached, a drop of bromcresol purple was added and neutralization continued until there was a distinct color change. Final adjustment to pH 6.4 was made with 1 N NaOH, using the glass electrode; the solution was then made up to 100 cc. with water.

For test of the strength of the pyruvate solution, three Warburg vessels were required with the following contents, in cc.:

	(1)	(2)	(3)
Main compartment: Carboxylase.....	0.5	0.5	None
0.25 M phosphate, pH 6.4.....	0.3	0.3	None
Water.....	1.5	2.0	2.3
Side arm: pyruvate solution, diluted 1:100.....	0.5	None	0.5
Center cup: 2.5 N HCl.....	0.5	0.5	0.5

After a 10 minute period of equilibration at 30° C. the pyruvate was dumped and the side arms rinsed. Readings were taken until a constant value for  $\text{CO}_2$  evolved was obtained; this took up to 2 hours or more. The acid in the center cup was then mixed with the contents of the main compartment and the readings continued until a constant value was again obtained. The  $\text{CO}_2$  evolution from decomposition of pyruvate was given by the difference of (1) — (2) — (3). If the undiluted pyruvate solution had been made from pure pyruvic acid and no decomposition had occurred during neutralization, it should have been 2 M in strength; the value usually obtained here was 1.3–1.6 M.

*Preparation of Arbacia Egg Extracts and Their Assay for Diphosphothiamine.*—Ripe, mature eggs of *Arbacia punctulata* were obtained at Woods Hole during July, August, and early September, 1941. When necessary, they were fertilized and allowed to develop at 20° C. according to methods reported in previous papers of this series. The volume of the eggs was, in every case, determined on the unfertilized eggs by the hematocrit method (2700 times gravity for 5 minutes). Each cubic centimeter of

eggs corresponded to approximately  $5 \times 10^4$  eggs. The diphosphothiamine values per cubic centimeter of eggs were converted to a wet weight and a dry weight basis using an egg density of 1.09 (13) and an egg solid content<sup>2</sup> of 24 per cent (14). There is 0.1 mg. N per mg. dry weight (14).

Diphosphothiamine was determined at three stages in egg development at 20° C.: unfertilized; at 30 minutes after fertilization, by which time the eggs were in the sperm aster stage; and at 10 hours after fertilization, by which time the eggs had just hatched from their fertilization membranes and become swimming blastulae. In each case the eggs were allowed to develop, with occasional stirring, in a large volume of sea water contained in a large crystallizing dish; this was kept in a water thermostat. For analysis, the eggs were collected by sharp centrifuging.

Except where otherwise noted, the extraction fluid was 1 N NaCl, to which an appropriate amount of HCl or NaOH was added to give the final desired pH in the egg extract. Preliminary tests showed that most of the echinochrome could be removed, along with the denatured egg protein, if the salt solution was employed instead of water. Other agents, such as protein precipitants, non-aqueous solvents, and thiamine chloride were added to the extraction fluid as described under "Experimental results." In a few instances the eggs, suspended in the extraction fluid, were broken up by 1 to 2 minutes homogenizing in a Waring mixer but, since this procedure did not increase the yield of diphosphothiamine, it was subsequently abandoned.

The type procedure finally adopted and recommended for the extraction was as follows: Two 10 cc. samples of egg suspension, each containing about 0.5 gm. wet eggs, were transferred to 15 cc. graduated centrifuge tubes, by means of a pipette with a wide opening, and packed by centrifuging gently for 2 minutes. To the first sample, 5 cc. of extraction fluid were added without stirring up the eggs, and the tube placed in a boiling water bath. The fluid and eggs were mixed by means of a small stirring rod and the tube left in the water bath with constant mixing for a total of 3 minutes. The tube was then cooled and centrifuged at 2700 gravity for 3 minutes. The supernatant fluid was transferred to another graduated tube. A fresh 5 cc. of extracting fluid were added to the precipitate, the tube kept in the boiling water bath for 3 minutes, cooled, and centrifuged as described above. The resulting supernatant fluid was mixed with that obtained during the first extraction, and the combined volumes of extract and precipitate noted. If necessary, the extract was brought to pH 6.4–6.7 for the test. The total volume of extract was taken as the sum of the volumes of egg residue, combined egg extracts, and solution added for pH adjustment. This total volume was usually 10 to 12 cc. Control experiments showed that a single 5 cc. or 10 cc. extraction was inadequate and that the yield was not improved by increasing the number of extractions beyond the second. The second sample was used for recovery of added diphosphothiamine; it was extracted with a previously prepared mixture of 5 cc. extracting fluid plus 0.1 cc. of a solution containing a known amount (usually 1 to 5 micrograms) of added synthetic diphosphothiamine. The mixture was added to the packed eggs immediately before transfer

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<sup>2</sup> The previously used value of 18 per cent solids, taken from older work, has since been found to be incorrect and has therefore been replaced by the higher value here employed, which was obtained for eggs handled in the same manner as the eggs used for the present experiments (14).

to the boiling water bath. 5 cc. of extracting fluid were added to the tube in which the original mixture was prepared, this tube thoroughly rinsed, and the resulting solution used for the second extraction.

The egg extracts were assayed for diphosphothiamine as follows: The main compartment of each Warburg flask received 0.3 cc. split carboxylase, 0.3 cc. Mn solution (410 mg.  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  per 100 cc. water), 0.5 cc. egg extract, 0.2 cc. 0.25 M phosphate buffer, pH 6.4, and 1.5 cc. water. The side arm received 0.5 cc. 1.6 M pyruvate. The materials were pipetted into the flasks *in the order given*, and the contents of the flask were mixed thoroughly after each addition. The vessels were equilibrated at 30° C. with air as gas phase. After 5 minutes the stopcocks were closed, and after 10 minutes total equilibration time the contents of the side arm were dumped and readings taken every 3 minutes for 18 minutes; the 3 minute reading was taken as zero time and the rate of gas evolution determined from the slope of the  $\text{CO}_2$  evolution-time curve.

The amount of diphosphothiamine present in each unknown solution was determined by comparison with a calibration curve obtained with a series of flasks in which the egg extract had been replaced with the same volume of an aqueous solution containing from 0.1 to 0.5 microgram of synthetic diphosphothiamine. Over a 12 to 15 minute period, the  $\text{CO}_2$  evolution was linear with time at concentrations of synthetic diphosphothiamine up to 0.4 to 0.5 microgram per flask. Likewise, the rate of  $\text{CO}_2$  evolution per unit time was linear with respect to diphosphothiamine concentration up to 0.4 microgram of synthetic diphosphothiamine per flask; the volume of egg extract was adjusted to make the amount of diphosphothiamine per flask fall within this range. Under the conditions stated, the average  $\text{CO}_2$  evolution (in excess of the blank) was approximately 150 to 200 c. mm. per hour per 0.1 microgram of synthetic diphosphothiamine, the rate varying somewhat with different split carboxylase preparations, but remaining constant for a week or more with any single preparation. A new calibration curve was run with each fresh split carboxylase preparation, and one or two points were checked at frequent intervals. Complete analyses in duplicate agreed within  $\pm 8$  per cent. From fourteen analyses carried out by the final extraction method, the recovery of diphosphothiamine was 92 per cent with a standard deviation of 7.9.

The diphosphothiamine standard was a sample of synthetic cocarboxylase from Merck and Co., Inc. (lot No. 41811), containing 12.64 per cent phosphorus and 11.74 per cent nitrogen (theory for  $\text{C}_{12}\text{H}_{19}\text{O}_7\text{N}_4\text{SClP}_2 \cdot \text{H}_2\text{O}$ , 12.96 per cent P and 11.68 per cent N). Through the kindness of Dr. R. A. Peters, who provided a sample of synthetic cocarboxylase (Merck lot No. 32079) which had been found in his laboratory to have 60 per cent of the activity of Lohmann and Schuster's (15) natural cocarboxylase from yeast, it was possible to determine the absolute activity of the sample here employed. By the above described manometric test, sample 41811 had, within the limits of experimental error, the same activity as sample 32079, thus agreeing in activity with the two samples of synthetic cocarboxylase which Weil-Malherbe (16) compared with the natural substance. On the basis of these results, the diphosphothiamine values, as determined in terms of sample 41811, were multiplied by 0.6 to convert them to a natural yeast cocarboxylase basis; the values given in the text and tables are in terms of natural cocarboxylase and would be 1.67 times as large if expressed in terms of synthetic sample 41811.

TABLE I

*Apparent Diphosphothiamine\* Content of Arbacia Eggs in the Unfertilized States (U), and After 30 Minutes (0.5 F) and 10 Hours (10 F) of Development at 20°C., as Determined After Extraction of the Eggs at Various pH Values in the Absence of Added Thiamine Hydrochloride*

Exp. No.	Date	Stage of egg development	Extraction method†	pH of egg extract before neutralization	Wet weight eggs used	Diphosphothiamine found per gm. wet weight	Recovery of diphosphothiamine added to test system		Recovery of diphosphothiamine carried through entire extraction process	
							Added	Found	Added	Found
					mg.	μg.	μg.	per cent	μg.	per cent
21	7-10-41	U	1	3.0	990	1.2			0.59	0
24	7-14	U	1	3.0	1770	0.8	0.12	93	2.2	80
26	7-16	U	1	3.0	1430	1.3			2.2	54
		0.5 F	1	3.0	1430	1.2			2.2	0
		10 F	1	3.0	1430	1.2			2.2	0
28	7-17	U	2	3.0	1030	1.1			2.2	128
		0.5 F	2	3.0	1030	0.9			2.2	82
29	7-18	U	2	3.0	724	1.6			2.2	19
32	7-21	U	3	6.0	1195	0.6			2.3	67
33	7-21	U	4	3.0	1250	1.0	0.06	93	2.3	82
35	7-22	U	5	6.0	1210	2.7	1.6	110	2.3	94
35A	7-22	U	6	3.0	725	1.4	0.06	90	2.3	57
36	7-23	U	7	3.0	875	0.3	0.06	109	2.3	61
38	7-24	U	8	3.9	507	0.6	0.06	72	2.3	57
51	8-6	U	9	5.0	396	1.0	0.06	98	2.6	80
		0.5 F	9	5.4	396	1.1	0.06	100	2.6	72
		10 F	9	5.1	396	0.6	0.06	100	2.6	77
56	8-7	U	10	4.0	298	0.4	0.06	107	2.6	82
		0.5 F	10	4.0	298	0.8			2.6	65
		10 F	10	4.0	298	1.3			2.6	66
66	8-12	U	11	4.8	560	0.5			2.7	71
		0.5 F	11	4.8	560	0.4			2.7	81
66A	8-12	U	12	4.2	510	0.7			2.7	76
70	8-13	U	13	4.9	536	0.3			2.7	71
		0.5 F	13	4.9	536	0.3			2.7	74

\* Referred to natural yeast cocarboxylase as standard. See "Experimental methods and materials."

† The extraction methods were as follows:

1. The packed eggs were taken up in 10 to 20 volumes 1 M NaCl which was 0.03 N in HCl, homogenized in the Waring mixer for 1 minute at high speed, heated 5 minutes, cooled, brought to pH 6.4, filtered.

2. Same as method (1), except for inclusion of 0.5 mg.  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  per cc. of extraction fluid; this procedure caused almost complete precipitation of echinochrome from the extracts.

3. The packed eggs were taken up in 20 volumes distilled water, homogenized in the Waring mixer for 1 minute at high speed, filtered.

4. Same as (1) without heating.

5. Same as (3), except that, after homogenizing in the Waring mixer, the egg brei was

## EXPERIMENTAL RESULTS

*Diphosphothiamine Content of Arbacia Eggs.*—It was found that both the naturally present and added diphosphothiamine were very difficult to recover quantitatively from either the heated or unheated egg extracts. The results of a number of preliminary extraction procedures are illustrated by the representative data given in Table I, from which it may be seen that the diphosphothiamine values varied from 0.3 to 1.6 micrograms per gram of wet eggs and the recovery of added diphosphothiamine varied, apparently with no consistent relationship to the diphosphothiamine content of the eggs, from 0 to 90 per cent.

Investigation of the recovery at various stages of the extraction process showed that added diphosphothiamine was not lost as a result of the phosphatase activity of the eggs, the degree of acidity used, the heat treatment employed, but by becoming bound to the egg residue. This is illustrated by a typical experiment:

5 identical aliquots of unfertilized eggs from the same batch were treated as follows: The first sample of packed eggs was taken up in 3 volumes of 1 *N* NaCl containing 0.03 *N* HCl, homogenized for 1 minute in a Waring mixer, heated 5 minutes in a boiling water bath, cooled, neutralized, and filtered; this was the reference sample upon which recovery calculations for the other four samples were based. The second, third, fourth, and fifth samples were carried through exactly like the first, except that known amounts of diphosphothiamine were added just before homogenizing, before heating, before filtering, and before test, respectively. The percentage recoveries were, respectively, 80, 72, 80, 105.

adjusted to pH 8 with NaOH and allowed to stand for 10 minutes at room temperature, then brought to pH 6.4 and filtered.

6. The packed eggs were taken up in 30 volumes 1 *M* NaCl which was 0.03 *N* in HCl, homogenized in the mixer for 1 minute at high speed; enough calgon (calcium hexameta-phosphate) to give 1 mg. per cc. was then added; the mixture stirred for 10 minutes and centrifuged. The supernatant extract was then adjusted to pH 6.4.

7. Same as (6) except that, after the calgon addition, the mixture was heated 5 minutes, allowed to stand 10 minutes, then centrifuged and the supernatant brought to pH 6.4.

8. Same as (1), except that extraction was made with 1 *M* NaCl which was 0.005 *N* in HCl.

9. The packed eggs were taken up in 5 cc. 1 *M* NaCl which was 0.005 *N* in HCl, heated 3 minutes, cooled, centrifuged; the supernatant liquid was transferred to another tube. This extraction was repeated with another 5 cc. of the same extraction fluid, the supernatant extracts combined, brought to pH 6.4, filtered.

10. Same as (9), except that first extraction was made with 1 *M* NaCl which was 0.008 *N* in HCl.

11. Same as (9), except that first extraction was made with 1 *M* NaCl which was 0.008 *N* in HCl, the second with 1 *M* NaCl 0.005 *N* in HCl.

12. Same as (9), except that both extractions were made with 1 *M* NaCl which was 0.05 *M* in sodium acetate and 0.05 *N* in HCl.

13. Same as (9) except that each extraction was made at 70°C. for 5 minutes instead of in a boiling water bath for 3 minutes.

Thus, there was essentially complete recovery at every stage except the filtration, where the diphosphothiamine was subject to no loss except by binding to the egg residue or to the sodium tungstate-precipitable material left in the heated egg extracts. Use of calgon (calcium hexametaphosphate) as a precipitating agent was unsuccessful, as was removal of protein material by warm 50 per cent acetone; reduction of the amount of diphosphothiamine added for recovery and variation in egg extraction fluid ratio also failed to give higher recoveries (Table I). It was later found that recoveries ranging from 80 to 104 per cent (the average was 92 per cent with a standard deviation of 7.9) could be obtained by a combination of two procedures: (a) addition of 40 micrograms per cc. of thiamine hydrochloride (*i.e.* vitamin B<sub>1</sub> hydrochloride or thiamin chloride) to the extraction fluid, and (b) adjustment of the extraction pH to a value of 6.3–6.7; neither procedure was effective when used separately.

The use of thiamine hydrochloride was suggested by the observation of Ochoa and Peters (8), confirmed by Westenbrink and coworkers (17), that thiamine hydrochloride could stimulate the activity of a relatively crude yeast carboxylase preparation. Though this effect was attributed by these authors to inhibition of phosphatase activity, it seemed possible, in view of the fact that the added diphosphothiamine was in contact with the unheated eggs for only a few seconds at most, that the thiamine hydrochloride might produce this effect in the egg extracts by displacing diphosphothiamine from linkages to catalytically inactive protein, thus enabling the diphosphothiamine to combine with the catalytically active protein to enhance the overall activity of the decarboxylating enzyme.

With this possibility in mind, the effect of thiamine hydrochloride on the effectiveness of diphosphothiamine extraction from the eggs and on the activity of egg extracts was tested. A typical experiment is summarized in Table II. It was found: (a) that thiamine hydrochloride did not stimulate the activity of the present samples of split carboxylase when these were supplied with pure diphosphothiamine; (b) that the catalytic activity of a given amount of diphosphothiamine in the *egg extracts* could be greatly enhanced by inclusion of a small concentration of thiamine hydrochloride in the manometric assay system, this effect reaching a maximum at about 5 to 10 micrograms per flask (*i.e.*, 1.6 to 3.2 micrograms per cc. of assay system); (c) that addition of thiamine hydrochloride directly to the extraction fluid before its addition to the eggs also increased the amount of diphosphothiamine found in the assay; and (d) that the effect of adding 40 micrograms of thiamine hydrochloride to each cubic centimeter of extract was the same, regardless of whether the addition was made to the original extraction fluid or to the system directly before assay of the extract. These observations appear to cast some light on the variable diphosphothiamine values obtained by the methods summarized in Table I, since they indicate that the amount of diphosphothiamine found by assay in a given egg sample

depends on the amount of free (non-phosphorylated) thiamine also present. Preliminary analyses by the thiochrome method (18) indicated that the eggs contain about one-tenth to one-half as much free thiamine as diphosphothiamine, the ratio varying with individual egg samples; this would provide amounts of thiamine hydrochloride ranging up to a few tenths of a microgram per flask. Such amounts of thiamine, if present in certain extracts and absent in others, might cause considerable variation in apparent yields of diphospho-

TABLE II

*Effect of Thiamine Hydrochloride on Carboxylase Activity of Test System Formed from Split Carboxylase and Arbacia Egg Extracts\**

Thiamine hydrochloride added to test system  μg. per 3.3 cc.	CO <sub>2</sub> evolution per 15 min. per 3.3 cc. test system containing:			
	0.5 cc. Extract 1	0.5 cc. Extract 2	0.5 cc. Extract 3	0.5 cc. Extract 4
	c. mm.	c. mm.	c. mm.	c. mm.
0	54	95	101	141
1	85	127	101	151
2	89	160	105	144
5	94	140	96	153
20	98	140	100	143
40	99	140	100	144

\* Extract 1: 490 mg. packed eggs were taken up in 5 cc. 1 M NaCl which was 0.003 N in NaOH, heated 3 minutes, cooled, centrifuged; the supernatant liquid was transferred to another tube. This extraction was repeated with another 5 cc. of the same extraction fluid; the supernatant extracts were combined (pH 6.5), giving a total volume of 9.7 cc. Of this, 0.5 cc. was put into each test flask and run as described under "Experimental methods".

Extract 2: Same as extract 1, except that 2.33 micrograms of synthetic diphosphothiamine (equivalent to 1.40 micrograms natural cocarboxylase) were added to the first 5 cc. portion of extraction fluid.

Extract 3: Same as extract 1, except that the extraction fluid contained 40 micrograms of thiamin chloride per cc.

Extract 4: Same as extract 2, except that the extraction fluid contained 40 micrograms of thiamin chloride per cc.

thiamine by the manometric method in the absence of added thiamine hydrochloride.

The results of a number of analyses for diphosphothiamine, using 40 micrograms of thiamine hydrochloride per cc. of extraction fluid, are given in Table III. This method of extraction, when carried out between pH 2 and pH 6, gave consistent values for the diphosphothiamine content of the eggs, but low recoveries. Only when the extraction in the presence of added thiamine hydrochloride was carried out at pH 6 to pH 7 were recoveries of 80 to 100 per cent consistently achieved. Extraction at higher pH values, like those previously mentioned at low pH, gave lower values for absolute diphosphothiamine content and lower recoveries.



It was also found (Table III) that the apparent diphosphothiamine content of the eggs was highest in the unfertilized eggs, somewhat smaller at 30 minutes after fertilization, and still smaller at 10 hours after fertilization. Whether this decrease was due to a loss of diphosphothiamine during the development of the eggs, to a progressively tighter binding and less complete extraction of the diphosphothiamine of the eggs, or to other causes, cannot be determined from the present data.

*Preliminary Experiments on Pyruvate Utilization by Arbacia Eggs and Egg Extracts.*—While the present experiments were in progress, it was reported by Barron and Goldinger (19), using a colorimetric method for pyruvate determination, that both fertilized and unfertilized *Arbacia* eggs could use pyruvate, the rates of pyruvate disposal by fertilized and unfertilized eggs being 354 micrograms and 70 micrograms per hour per gm. dry weight, respectively. They also found eggs of *Arbacia* to contain about 850 micrograms of pyruvic acid per gm. dry weight and 2.72 micrograms of diphosphothiamine per cc. of packed eggs.

In the course of the work here described, two sets of observations bearing on the use of pyruvate have been made. Though these experiments were intended to be purely preliminary, they are reported here because an opportunity to extend and complete them may not be available for a considerable time.

1. Parallel samples of egg cytolysate, obtained by mixing 1 volume of packed eggs with 3 volumes of water, were shaken without or with added pyruvate for 2 hours at 20°C. in Warburg vessels. The pyruvate present at zero time and after 2 hours was determined by the carboxylase method as described above.<sup>3</sup> Each experiment was run in duplicate or triplicate. Control experiments gave 99 per cent recovery of added pyruvic acid by this method. Though the results, because of the small changes in pyruvate and the limited number of experiments, must be regarded as only suggestive, two points were indicated.

In the first place, from six experiments on pyruvate utilization under aerobic conditions (Table IV) cytolysates from 30 minute fertilized eggs used, on the average, about 310 micrograms pyruvate per gm. dry weight per hour ( $1.7 \times 10^{-6}$  moles or 75 micrograms per gm. wet weight), while cytolysates from unfertilized eggs used about 160 micrograms pyruvate per hour per gm. dry weight ( $0.87 \times 10^{-6}$  moles or 38 micrograms per gm. wet weight). These results tend, therefore, to confirm in principle the experiments of Barron and

<sup>3</sup> All  $\text{CO}_2$  obtained as the result of adding carboxylase is here calculated as pyruvic acid, though carboxylase can (9) liberate  $\text{CO}_2$ , at a rate much smaller than from pyruvic acid, from other possible intermediary metabolites such as oxalacetic and  $\alpha$ -ketoglutaric acids. At the first opportunity it is proposed to employ both the colorimetric pyruvate method and the carboxylase method for analysis of the eggs. This may give some indication of the extent to which  $\alpha$ -keto acids other than pyruvic contribute to the values here obtained.

TABLE III

*Apparent Diphosphothiamine\* Content of Arbacia Eggs in the Unfertilized State (U), and after 30 Minutes (0.5 F) and 10 Hours (10 F) of Development at 20° C., as Determined after Extraction of the Eggs at Various pH Values in the Presence of 40 Micrograms per Cc. Thiamine Hydrochloride Added to the Extraction Fluid*

Exp. No.	Date	Stage of egg development	Extraction method†	pH of egg extract before neutralization	Wet weight eggs used	Diphosphothiamine found per gm. wet weight	Recovery of diphosphothiamine carried through entire extraction process	
							Added	Found
					mg.	µg.	µg.	per cent
87	8-20-41	U	14	2.2	575	2.5	2.7	83
		0.5 F	14	2.2	575	1.6	2.7	88
		10 F	14	2.1	575	1.9	2.7	74
100	8-29	U	14	1.8	360	2.5	2.8	74
		0.5 F	14	1.8	360	1.7	2.8	77
		10 F	14	1.8	360	1.2	2.8	82
103	8-30	U	14	1.9	370	3.2	2.8	65
		0.5 F	14	1.9	370	2.5	2.8	71
		10 F	14	1.8	370	1.8	2.8	70
105	9-1	U	14	2.0	522	2.5	2.8	66
		0.5 F	14	2.1	522	1.6	2.8	79
		10 F	14	2.0	522	1.3	2.8	68
107	9-2	U	14	1.6	386	2.9	2.8	53
108	9-2	U	14	1.7	418	2.2	2.8	64
		U	14	1.7	418	1.9	0.7	66
110	9-3	U	15	4.8	438	2.7	1.4	53
		U	16	5.8	438	1.9	1.4	75
112	9-4	U	17	6.0	495	2.9		
		U	18	8.7	495	2.7		
114	9-4	U	19	6.5	430	2.1	1.4	88
		U	18	8.9	430	1.7	1.4	81
117	9-5	U	19	6.4	432	2.0	1.4	82
		0.5 F	19	6.4	432	1.9	1.4	96
		10 F	19	6.4	432	1.0	1.4	92
118	9-5	U	19	6.5	439	2.0	1.4	80
		0.5 F	19	6.4	439	1.5	1.4	101
		10 F	19	6.3	439	1.7	1.4	97
120	9-6	U	19	6.6	374	2.3	1.4	90
		0.5 F	19	6.6	374	2.1	1.4	90
		10 F	19	6.6	374	1.8	1.4	104
121	9-6	U	19	6.5	376	3.4	1.4	92
		0.5 F	19	6.6	376	1.8	1.4	93
		10 F	19	6.6	376	1.3	1.4	104

\* Referred to natural yeast cocarboxylase as standard. See "Experimental methods and materials."

† The extraction methods, numbered to follow those of Table I, were as follows:

14. The packed eggs were taken up in 5 cc. 1 M NaCl which was 0.03 N in HCl and contained 40 micrograms thiamine hydrochloride per cc.; the mixture was heated 3 minutes, cooled, centrifuged, and the supernatant fluid was transferred to another tube. This extraction was repeated with another 5 cc. of the same extraction fluid, the supernatant extracts combined, brought to pH 6.4, filtered.

15. Same as (14), except that extraction fluid was 0.005 N in HCl.

16. Same as (14), except that extraction fluid contained no added HCl.

17. Same as (14), except that extraction fluid was 0.001 N in NaOH.

18. Same as (14), except that extraction fluid was 0.01 N in NaOH.

19. Same as (14), except that extraction fluid was 0.003 N in NaOH.

Goldinger. It may be calculated, without intending further implications, that the observed rates of pyruvate disposal, if expressed as complete oxidation to  $\text{CO}_2$  and water, would be enough to account for approximately one-third

TABLE IV

*Change in the Pyruvate Content of the Cytolysate from 0.5 Cc. Arbacia Eggs during Incubation under Aerobic or Anaerobic Conditions for 2 Hours at 20° C.*

Exp. No.	Cytolysate from unfertilized (U) or 30 min. (F) fertilized eggs	Without (-) or with (+) 0.005 M added pyruvate	Gas phase during incubation	CO <sub>2</sub> liberated by carboxylase from pyruvate in egg cytolysate		Change in pyruvate content of egg cytolysate during incubation	
				Before incubation	After 2 hrs. incubation	CO <sub>2</sub> equivalent	Pyruvate per hr. per wet gm. of eggs
				c. mm.	c. mm.	c. mm.	moles per l. $\times 10^3$
127	U	+	Air	193	188	-5	-0.2
	F	+	Air	211	184	-27	-1.2
128	U	+	Air	212	206	-6	-0.3
	F	+	Air	238	204	-34	-1.5
131	U	+	Air	250	227	-23	-1.0
	F	+	Air	287	221	-66	-2.9
132	U	+	Air	218	194	-24	-1.1
	F	+	Air	231	210	-21	-0.9
141	U	-	Air	31	0	-31	-1.4
	F	-	Air	42	0	-42	-1.9
	U	+	Air	193	144	-49	-2.2
	F	+	Air	198	159	-39	-1.7
133	U	+	N <sub>2</sub> *	245	221	-24	-1.1
	U	+	Air	245	211	-34	-1.5
135	F	+	N <sub>2</sub>	270	300	+30	+1.3
	F	+	Air	270	245	-25	-1.1
136	F	-	N <sub>2</sub>	83	120	+37	+1.6
	F	-	Air	83	38	-45	-2.0
	F	+	N <sub>2</sub>	298	285	-13	-0.6
	F	+	Air	298	264	-34	-1.5
143	F	+	Air	244	238	-6	-0.3
	F†	+	Air	244	228	-16	-0.7

\* The nitrogen was purified by passage through a 20 inch column of freshly reduced copper turnings.

† This sample contained 20 micrograms of added synthetic diphosphothiamine (equivalent to 12 micrograms natural cocarboxylase) per cc.

of the respiration of the fertilized egg and all of the respiration in the unfertilized egg.

In the second place, the net rate of pyruvate disappearance, both with the cytolysate from unfertilized and the cytolysate from fertilized eggs, was greater under aerobic than under anaerobic conditions. In two samples of

cytolysate from fertilized eggs, pyruvate appeared to be formed under anaerobic conditions.

2. Unfertilized or fertilized eggs, suspended as 50 per cent suspensions in sea water buffered at pH 8.0 with 0.05 M glycylglycine buffer, were incubated aerobically for 2 hours at 20°C., the fertilized samples being started at 30 minutes after fertilization. Under these conditions, with adequate shaking in the Warburg bath, the fertilized eggs developed at the same rate as in dilute suspension, 90 to 100 per cent being found in the 8-cell stage ready to proceed to the 16-cell stage at the end of the incubation period. Duplicate samples of eggs were cytolized by adding an equal volume of distilled water at zero time. The total pyruvate in the eggs and their suspension medium was determined by the carboxylase method. Parallel duplicate samples were subjected to the same treatment at the end of the 2 hour incubation period. In two experiments, with 0.005 M sodium pyruvate present in the suspension medium, the results indicated a net formation of products decomposed to CO<sub>2</sub> by carboxylase. The average of two determinations, each in duplicate, on unfertilized eggs was  $1.0 \times 10^{-6}$  moles pyruvate<sup>4</sup> formed per gm. wet eggs per hour; the corresponding value for the fertilized eggs, was  $1.1 \times 10^{-6}$  moles per gm. wet eggs per hour.

#### DISCUSSION

The data on recovery of added diphosphothiamine, presented above in Tables I and III, provide an interesting commentary on the value of recovery data in assessing the validity of analyses for various cellular constituents. These experiments show very clearly that, though incomplete recoveries are, in some instances, cause for rejection of analyses, complete recoveries cannot be accepted as proof that all of the substance sought is actually extracted from the biological material in which it naturally occurs. It should be stated, therefore, that, in spite of the constancy of analytical values obtained with various samples of eggs and the virtually complete recovery of added diphosphothiamine,<sup>4</sup> the present estimate of the absolute amount of this substance in *Arbacia* eggs may be subject to upward revision if more effective extraction methods should be developed subsequently.

Whatever the absolute diphosphothiamine content of *Arbacia* eggs may ultimately prove to be, the present experiments show quite conclusively that the amount of this substance in the eggs is comparable with that in other animal tissues. This suggests that diphosphothiamine, and the processes catalyzed by it, may play a prominent rôle in the aerobic metabolism of the eggs. Two further lines of evidence are available to support this suggestion.

First, as reported by Barron and Goldinger (19) and confirmed by the

<sup>4</sup> The same reservations are applicable to the diphosphopyridine nucleotide data to be presented in the following paper (20).

preliminary experiments presented in this paper, the eggs have a mechanism for metabolizing pyruvic acid; this disposal of pyruvic acid depends, in some degree at least, on the presence of oxygen, being apparently somewhat more rapid under aerobic than under anaerobic conditions.

Secondly, as is shown in another paper of this series (14), the eggs can obtain energy for development either by oxidation of carbohydrate or by oxidative deamination; in either case, adequate precursors for formation of pyruvic and other  $\alpha$ -ketonic acids are available. Indeed, it has been found in the present experiments that developing *Arbacia* eggs can, under certain conditions, apparently form pyruvic acid. Detailed elucidation of the processes of pyruvic acid formation and disposal—as well as the relation of diphosphothiamine, suitable phosphate acceptors, and other factors to these processes—requires further experiment.

The authors wish to thank Dr. R. A. Peters for providing a reference sample of cocarboxylase. They also wish to thank Miss A. K. Keltch for performing the thiamine analyses referred to above, and Mr. L. A. Baker for assistance in preparing the eggs for analysis.

#### SUMMARY

1. Methods suitable for the determination of diphosphothiamine (cocarboxylase) in eggs of *Arbacia punctulata* have been developed. Quantitative extraction of the cocarboxylase was effected by combining the use of thiamine hydrochloride in the extraction fluid with critical adjustment of the pH of extraction to pH 6.3–6.7.

2. The unfertilized eggs were found to contain the equivalent of 2 to 3 micrograms of natural yeast cocarboxylase per gm. of wet eggs; the cocarboxylase content of the 30 minute and 10 hour fertilized eggs was somewhat less (Table III).

3. In preliminary experiments, *Arbacia* egg cytolysates were found to cause pyruvic acid to disappear. The rate of such disappearance was apparently greater under aerobic than under anaerobic conditions; it was also greater for cytolysates from fertilized eggs than for cytolysates from unfertilized eggs (Table IV).

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## STUDIES ON CELL METABOLISM AND CELL DIVISION

### VIII. THE DIPHOSPHOPYRIDINE NUCLEOTIDE (COZYMASE) CONTENT OF EGGS OF *ARBACIA PUNCTULATA*

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This paper deals with the occurrence of diphosphopyridine nucleotide in *Arbacia* eggs. This substance acts as a coenzyme in numerous aerobic and anaerobic metabolic processes (see references 1 and 2 for summaries of its functions). It has also been shown to be involved in at least one type of oxidative energy transfer, namely, the coupling of phosphorylation with oxidation of triosephosphate (3-5). It was therefore of interest to find, in the course of a systematic survey of possible energy-yielding processes in *Arbacia* eggs, that the diphosphopyridine nucleotide content of these eggs, 250-500 micrograms per gram wet weight, is comparable with that of other animal tissues and of certain yeasts (6-8).

#### *Experimental Methods and Materials*

*Preparation of Arbacia Egg Extracts and Their Assay for 'Diphosphopyridine Nucleotide'.*—Eggs of *Arbacia punctulata* were obtained at Woods Hole during July, August, and early September, 1941. They were handled, and the results expressed, exactly as described in the previous paper (9). The type procedure for extraction of the diphosphopyridine nucleotide was the same as for diphosphothiamine, with the modifications shown in the footnotes to Table I of the present paper. The total volume of the egg extract was taken as the sum of the volume of the combined supernatant fluids after centrifuging off the egg residue, and the volume of fluid added for pH adjustment; control experiments indicated that the amount of diphosphopyridine nucleotide left in the egg residue after the second extraction could be disregarded. For each determination, two samples of eggs from the same batch were run; one sample was used to determine the diphosphopyridine nucleotide content of the eggs themselves; the other was used for recovery of added diphosphopyridine nucleotide by the same procedure as previously described for diphosphothiamine (9).

The extracts were assayed for diphosphopyridine nucleotide by the method of Jandorf, Klemperer, and Hastings (10), which depends upon the enzymatic conversion of hexosediphosphate into phosphoglyceric and glycerophosphoric acids under conditions such that the rate of conversion is proportional to the concentration of diphosphopyridine nucleotide present. The amount of acid produced in a given time is measured manometrically by following the CO<sub>2</sub> liberated from a bicarbonate buffer.

In the present experiments, the main compartment of each Warburg flask received



TABLE I

*Diphosphopyridine Nucleotide Content of Arbacia Eggs in the Unfertilized State (U) and after 30 Minutes (0.5 F) and 10 Hours (10 F) of Development at 20° C.*

Exp. No.	Date	Stage of egg development	Extraction method*	pH of egg extract before neutralization	Wet weight eggs used	Diphosphopyridine nucleotide found per gm. wet weight	Recovery of diphosphopyridine nucleotide carried through entire extraction process	
							Added	Found
					mg.	μg.	μg.	per cent
42	7-28-41	U	1	4.6	362	255	59	22‡
		0.5 F	1	4.6	362	279	59	61‡
		10 F	1	4.4	362	282	59	30‡
49	8-4	U	1	5.0	422	416	59	95
		0.5 F	1	5.2	422	364	59	100
		10 F	1	4.9	422	248	59	85
55	8-7	U	1	4.1	336	412	81	106
		0.5 F	1	4.0	336	372	81	92
		10 F	1	3.7	336	289		
60	8-9	U	1	5.2	870	417	75	100
		0.5 F	1	5.3	870	365	75	94
		10 F	1	5.2	870	285	75	93
67	8-13	U	2	4.8	560	446	99	93
		0.5 F	2	4.8	560	294	99	97
67A	8-13	U	3	4.2	510	317	99	85
71	8-13	U	4	4.9	536	390	75	91
		0.5 F	4	4.9	536	388	75	90
		U	5	4.9	536	308	75	77
75	8-14	U	6		453	329	83	90
78	8-15	U	7	3.8	274	453	38	108
		U	8	8.2	274	352	38	0
		0.5 F	7	4.1	274	274	38	105
		0.5 F	8	7.4	274	358	38	58
		10 F	7	3.7	274	170	38	102
		10 F	8	8.7	274	217		
		U	9	2.2	554	392	122	90
84	8-18	U	10	12	554	134		
		0.5 F	9	2.3	554	284	122	97
		0.5 F	10	12	554	101		
		10 F	9	2.1	554	294	122	99
		10 F	10	12	554	193		
		U	9	2.2	575	360	122	91
		U	10	12	575	102		
88	8-20	0.5 F	9	2.3	575	267	122	94
		0.5 F	10	12	575	123		
		10 F	9	2.1	575	236	122	100
		U	9	1.8	360	184	72	99
		U	10	12	360	68		
		0.5 F	9	1.8	360	188	72	106
		0.5 F	10	12	360	53		
101	8-29	10 F	9	1.8	360	186	72	104
		10 F	10	12	360	45		
		10 F	10	12	360			

TABLE I—*Concluded*

Exp. No.	Date	Stage of egg development	Extraction method*	pH of egg extract before neutralization	Wet weight eggs used	Diphosphopyridine nucleotide found per gm. wet weight	Recovery of diphosphopyridine nucleotide carried through entire extraction process	
							Added	Found
					mg.	μg.	μg.	per cent
104	8-30	U	9	1.8	370	334	72	97
		U	10	12	370	150		
		0.5 F	9	1.8	370	300	72	87
		0.5 F	10	12	370	208		
		10 F	9	1.8	370	219	72	91
		10 F	10	12	370	75		
106	9-1	U	9	2.1	522	376	72	106
		0.5 F	9	2.1	522	284	72	94
		10 F	9	2.1	52	252	72	100

\* The extraction methods were as follows:

1. The packed eggs were taken up in 5 cc. 1 M NaCl which was 0.005 N in HCl, heated 3 minutes in a boiling water bath, cooled, centrifuged; the supernatant liquid was transferred to another tube. This extraction was repeated with another 5 cc. of the same extraction fluid, the supernatant extracts combined, brought to pH 6.4, filtered.

2. Same as (1), except that the first extraction was made with 1 M NaCl which was 0.008 N in HCl, the second with 1 M NaCl 0.005 N in HCl.

3. Same as (1), except that both extractions were made with 1 M NaCl which was 0.05 M in sodium acetate and 0.05 N in HCl.

4. Same as (1), except that both extractions were made at  $70 \pm 1^\circ \text{C}$ . for 5 minutes instead of in a boiling water bath for 3 minutes.

5. Same as (4), except that  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  to give 0.05 mg. per cc. was added to the combined extracts. The pH was then brought to pH 7.4, and the precipitated echinochrome filtered off.

6. The packed eggs were extracted twice in a water bath at  $50 \pm 1^\circ \text{C}$ . with 5 cc. of 0.005 N HCl in 50 per cent acetone. After centrifuging off the precipitated material, the combined supernatant solutions were approximately neutralized. After standing in the cold for 30 minutes, a heavy brown precipitate had formed; this was centrifuged off and discarded. The supernatant was aerated for 30 minutes at  $45\text{--}50^\circ \text{C}$ ., which reduced the volume to 5.2 cc. The solutions were brought to 10.0 cc. with 2 M NaCl, adjusted to pH 6.5–6.6, centrifuged to remove a little more echinochrome.

7. Same as (1), except that both extracts were made with 2.5 cc. 1 M NaCl which was 0.01 N in HCl.

8. Same as (1), except that both extracts were made with 2.5 cc. 1 M NaCl which was 0.01 N in NaOH.

9. Same as (1), except that both extracts were made with 1 M NaCl which was 0.03 N in HCl.

10. Same as (1), except that both extracts were made with 1 M NaCl which was 1 N in NaOH.

‡ In these samples, the added diphosphopyridine nucleotide was allowed to stand in contact with the packed eggs before addition of extraction fluid; the low recoveries suggest that the unheated whole eggs have, in these three samples, caused some breakdown of the diphosphopyridine nucleotide. Accordingly, in subsequent experiments the diphosphopyridine was added with the extraction fluid and the mixture then brought to the temperature of the boiling water bath within a few seconds after the addition.

0.4 cc.  $\text{NaHCO}_3$  (0.154 M, saturated with 5 per cent  $\text{CO}_2$  : 95 per cent  $\text{N}_2$ ), 0.6 cc. of sodium hexosediphosphate (approximately 0.016 M), 0.3 cc. of  $\text{Na}_2\text{HAsO}_4$  (0.003 M), and 1.0 cc. of muscle extract, equivalent to 30 mg. of the muscle acetone powder. The egg extracts to be tested (0.02 to 0.1 cc. containing 1 to 4 micrograms diphosphopyridine nucleotide) were introduced into the side arm with a micro pipette, and water was added to the main compartment to make a total liquid volume of 3.0 cc. The manometers were filled in the bath with 5 per cent  $\text{CO}_2$  : 95 per cent  $\text{N}_2$  and equilibrated for a total of 15 minutes at  $38^\circ\text{C}$ . The contents of the main vessel and the side arm were mixed; 5 minutes were allowed to elapse for restoration of equilibrium, and manometer readings were then taken at 5 minute intervals for 30 minutes. The rate of gas evolution was determined from the slope of the  $\text{CO}_2$  evolution-time curve.

The amount of diphosphopyridine nucleotide present in each unknown sample was determined by comparison with a calibration curve obtained with a series of flasks in which the egg extract had been replaced with the same volume of an aqueous solution containing from 1 to 5 micrograms of diphosphopyridine nucleotide. Under the present experimental conditions the average  $\text{CO}_2$  evolution (in excess of the blank) was approximately 25 c.mm. per hour per microgram of the working standard sample of diphosphopyridine nucleotide, the rate varying somewhat with different muscle extract preparations, but remaining constant for more than a week with any given preparation. Owing to lack of suitable equipment for shaking the muscle extract during removal, by charcoal, of the diphosphopyridine nucleotide initially present in the acetone powder, the blank values were larger than those observed by Jandorf, Klemperer, and Hastings, being here approximately 15–17 c.mm. per hour per manometric flask. Calibration curves were run frequently, usually with each determination, using a freshly diluted diphosphopyridine nucleotide solution as reference.

The diphosphopyridine nucleotide working standard was prepared from yeast by the method of Jandorf (11). It had a purity of  $51 \pm 2$  per cent, as determined by comparison, in the manometric system, with a reference sample of diphosphopyridine nucleotide which had been shown to have a purity of 92 per cent on the basis of dithionite reduction and ultraviolet absorption.

From thirty-three analyses carried out for oxidized diphosphopyridine nucleotide, the recovery of diphosphopyridine nucleotide was 96 per cent with a standard deviation of 6.9.

#### EXPERIMENTAL RESULTS

*Diphosphopyridine Nucleotide Content of Arbacia Eggs.*—In contrast to the results reported for diphosphothiamine in the previous paper, it was found that both the naturally present and the added diphosphopyridine nucleotide were relatively easy to recover quantitatively. The results obtained with a number of extraction procedures are given in Table I. With the exception of one experiment (No. 42) where a relatively concentrated solution of the diphosphopyridine nucleotide was allowed to stand in contact with the packed eggs for some minutes before addition of extraction fluid and heating, the recoveries with acid extraction fluids were satisfactory.

Very early in the analyses it was observed that the apparent diphospho-

pyridine nucleotide content of the eggs tended to drop somewhat by 30 minutes after fertilization and still more by the 10th hour after fertilization. It seemed possible that this apparent decrease might be due to conversion, in the course of the egg development, of a part of the oxidized diphosphopyridine nucleotide to the dihydro form which would be destroyed during acid extraction (6).

To test this possibility, a number of parallel extractions were made, extracting at pH 2 to obtain only the oxidized form, and at pH 12 to obtain only the reduced form. Confirming Euler and coworkers (6), the oxidized diphosphopyridine nucleotide was rapidly destroyed in alkaline extraction fluids (No. 78, Table I), indicating that any activity recovered at pH 8 or above was associated only with the reduced form of the diphosphopyridine nucleotide. These experiments indicated that (a) the eggs have approximately 25–40 per cent of their total diphosphopyridine nucleotide in the reduced state, (b) the ratio of the reduced form to the total does not vary substantially during the first 10 hours of development, and (c) the value for total diphosphopyridine nucleotide as estimated from the sum of the separate values for oxidized and reduced forms is equal to or somewhat greater than that obtained by simultaneous extraction of both forms at a single pH in the range between pH 3.5 and pH 8.

*Attempts to Demonstrate Triosephosphate and Glutamic Dehydrogenases in the Arbacia Egg.*—Diphosphopyridine nucleotide is a component of both the triosephosphate (5) and the glutamic acid (12) dehydrogenase systems of animal tissues. Since fertilized *Arbacia* eggs can apparently consume both carbohydrate and amino acids during their early development (13), it would be of interest to know whether the eggs contain these two enzymes.

There are reasons for believing that triosephosphate dehydrogenase might not be detected in *Arbacia* egg cytolysates, even if the enzyme is present in the intact egg. In the first place, Rapkine (14) has shown that the activity of triosephosphate dehydrogenase from certain animal tissues is lost when the sulfhydryl groups of the enzymatically active protein are oxidized. In the second place, a number of naphthoquinones closely related to echinochrome have been found (15) to be excellent catalysts for oxidation of sulfhydryl groups. Thus, the echinochrome liberated from the pigment granules during cytolysis might seriously disturb tests for triosephosphate dehydrogenase and other enzyme systems in which activity is dependent on the presence of sulfhydryl groups in the reduced state.

Since the time and the egg supply required for a thorough investigation of this problem were not available at the end of the 1941 season, preliminary tests for triose dehydrogenase were carried out, at 20°C., on egg cytolysates in a system analogous to that employed in the diphosphopyridine nucleotide assay system and also by the ferricyanide method (16). Egg cytolysates equivalent to 500 mg. wet eggs were used in both cases. Tests of both types were negative, as were tests for glutamic acid dehydrogenase by the ferricyanide method.

It is proposed to continue the search for triosedehydrogenase in the echinochrome-free *Arbacia* egg fractions employed during the season of 1941 by Hutchens, Kopac, and Krah1 (17) for cytochrome oxidase determinations by the Cartesian diver method and, at the same time, to explore the possibility that control of the state of oxidation of sulfhydryl groups in certain enzymatically active proteins may provide one physiological function for echinochrome.

The authors are indebted to Mr. L. A. Baker for his assistance in preparing the eggs for analysis.

#### SUMMARY

1. The diphosphopyridine nucleotide content of *Arbacia* eggs has been measured manometrically and found to be approximately 250–500 micrograms per gm. wet weight of eggs, the value varying with individual egg samples and with the state of development of the eggs. Of the total diphosphopyridine nucleotide present, approximately 25–40 per cent is in an alkali-stable, presumably the dihydro, form.

2. Tests for triosephosphate and glutamic acid dehydrogenases carried out on *Arbacia* egg cytolysates were negative.

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# THE CHLOROPHYLL-PROTEIN COMPLEX

## I. ELECTROPHORETIC PROPERTIES AND ISOELECTRIC POINT\*†

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Although the chemical constitution and the physical properties of chlorophyll are well known, it is becoming increasingly clear that its behavior may be greatly modified through its attachment in the plant cell to a protein, which exists as a bearer. The notion of a chlorophyll-protein complex is not a new one. Beginning in 1884, with the suggestion of Reinke (1) that chlorophyll might exist in the form of a chlorophyll-protein complex very similar to hemoglobin, many authors (2-16) have from time to time supported the idea and, in some cases, have presented analytical evidence for its occurrence (10-12, 14, 16, 17). Some of this evidence was obtained with isolated chloroplasts (16, 17). In addition, French (17*a*) has investigated the pigment-protein complex from bacteria.

Until recently, methods for the isolation of the pigment-protein complex have been rather unsatisfactory. While a number of workers (5, 10-15) have indicated that it is possible to extract a protein, only Stoll and Wiedemann (12), Menke (10), and Smith (14) have been able to purify their preparations by fractionation with ammonium sulfate. In particular, Menke (10) has pointed out that he was able to separate the chlorophyll-protein complex from the cytoplasmic proteins.

Although it seems probable from nitrogen analyses, behavior with ammonium sulfate, and sedimentation studies (18) that the chlorophyll is actually attached to or bound with a protein into some sort of complex, it seemed desirable to obtain more information concerning the electrophoretic behavior of the complex in as unaltered a state as possible. It was particularly desirable to consider at least two unrelated species of plants. Such an investigation, aside from yielding information concerning the protein nature of the complex, should help explain the unusual behavior of the protein at various values of pH. In addition, it should indicate whether the protein complex is the same or differs from species to species.

In 1912, Herlitzka (19) subjected the press juice of leaves to electrophoresis

\* A preliminary note has already appeared (26).

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and noticed its migration to the anode. Later, Bakker (20) found that aqueous suspensions of chlorophyll, precipitated from acetone solutions and free from protein, were negatively charged but became positive on exposure to light for several hours. Katz and Wassink (21) reported that green aqueous extracts prepared from *Chlorella*, were negative at concentrations of HCl up to 0.001 N, ceased movement in 0.002 N HCl, and reversed in direction at slightly higher concentrations. Neish (17) observed that chloroplasts isolated from *Trifolium*, although negative in distilled water, were isoelectric at about pH 3.7. Shafer also reported that aqueous chlorophyll suspensions were negative, but the conditions were not clearly described. Aside from these brief reports, there has been no thorough investigation of the electrophoretic behavior of the chlorophyll-protein complex. Although much of the evidence in the literature was obtained by using isolated chloroplasts, the results to be reported here were obtained with highly purified preparations of the chlorophyll-protein complex.

### Methods

*Preparation of the Complex.*—Suspensions of the chlorophyll-protein complex were prepared by a modification of the methods of Menke (10) and Stoll and Wiedemann (12). Leaves of both *Aspidistra elatior* Blume and *Phaseolus vulgaris* L. (var. white navy) were used. The leaves were separated from the stems, washed free from excess dirt, and ground mechanically in a porcelain mortar, with M/100 phosphate buffer at pH 7.3–7.5. Sand was not used, lest the grinding disperse some of the sand particles colloiddally and interfere with the electrophoretic studies. The deep green suspensions obtained by grinding were filtered through muslin and then centrifuged for 30 minutes at top speed in a Swedish angle centrifuge to remove nuclear fragments and cellular debris. For the navy bean, the supernatant was made 1.3 M with respect to ammonium sulfate, which had been previously neutralized to pH 7.0. With *Aspidistra*, the molarity of the ammonium sulfate necessary to effect precipitation was somewhat higher, namely 1.9 M. This latter concentration agrees with that used by Smith (14). After about an hour, the suspensions were centrifuged at top speed for 30 minutes, whereupon the green material sedimented, leaving a yellow-brown supernatant. At times, the supernatant appeared slightly green in color after the first precipitation and centrifugation, indicating that not all of the chlorophyll-protein had been precipitated and that the concentration of salt used in the precipitation was minimal. Thus, even on the first precipitation, a large part of the impurities and foreign protein remained in the solution. While still moist, the green sediment was taken up in the phosphate buffer mixture and the operation of precipitation and suspension repeated twice more in the course of several hours. As Stoll and Wiedemann point out, a double repetition of the precipitating operation exhausts its effectiveness. The green material was finally resuspended in some of the original buffer mixture and dialyzed against the same buffer until free from sulfate. All preparative operations were carried out at 0°C., and in the absence of metal. No appreciable loss from denaturation was noticed.

When it was essential to have a preparation completely free from salt, the green sediment obtained on the final centrifugation was resuspended in distilled water (distilled in glass) and dialyzed until sulfate-free. This method produced no apparent change in the properties of the material.

At this point it may be well to point out that the concentration of ammonium sulfate necessary to effect precipitation of the chlorophyll-protein complex was markedly influenced by the time period over which the salting-out was allowed to take place. This agrees with the observations of Menke.

We also attempted to use a Waring blender but found that a large volume of foam was produced, with considerable denaturation, on the disruption of the leaf material. Furthermore, the material purified in this way not only precipitated quite rapidly during the course of several days, but in contrast with preparations made by other means the agglomerates failed to pass through ordinary filter paper.

*Electrophoresis.*—All measurements of electrophoresis were made in an Abramson type of microelectrophoresis cell following the techniques described in detail by Moyer (22). This method was especially suitable because of the particulate character of the purified suspensions. Unless otherwise stated, measurements were made in  $M/50$  acetate buffers at an ionic strength of 0.02. The mobilities, in  $\mu/\text{sec.}/\text{volt}/\text{cm.}$ , were corrected to 25°C. from room temperature by correcting for changes in viscosity. Both quinhydrone and glass electrodes were used to determine pH.

When the purified material was used, an aliquot of the concentrated suspension (0.25 to 0.50 cc.) was added to the buffer salt and the suspension brought to the desired volume and pH by the addition of acetic acid and water. When quartz or collodion particles were used, these were first suspended in the concentrated chlorophyll-protein solution for about 15 minutes to insure complete coating before addition of the buffer, as above. This procedure minimized the amount of clumping that resulted when the chlorophyll-protein complex was suspended in buffers on the acid side of its isoelectric point.

#### EXPERIMENTAL

*General Properties of the Complex.*—Purified suspensions of the chlorophyll-protein complex exhibit several interesting properties. They are deep green in color and exceedingly stable, remaining apparently unaltered over a long period of time, either at the pH of the phosphate buffer (pH 7.3–7.5), or, after dialysis against distilled water, at about pH 6.0. In addition, the suspensions exhibit the physical characteristics described by other workers (5, 12–15): a strong maximum absorption band at about 678  $m\mu$ , photostability, red fluorescence, and sensitivity to protein coagulants. On the other hand, either an increase in temperature, removal of water by drying, or freezing can denature the material. Smith (14) pointed out that flocculation of the chloroplast material from both *Aspidistra* and *Spinacia* takes place below pH 4.5. We have found that our purified extract flocculates almost instantaneously at and below the isoelectric point. Flocculation or denaturation on the acid side of the isoelectric point is not inhibited by carrying out the operations at 0°C.



Acetone or alcohol splits the complex. If ether is layered above the suspension, alcohol or acetone will cause the chlorophyll to pass into the ether phase, as the protein precipitates. Gentle shaking of the material with ether alone causes little, if any, of the chlorophyll to pass into the ether layer. Vigorous shaking, which splits the pigment-protein complex, results in a more intense coloring of the ether layer. This last result agrees with the observations of Stoll and Wiedemann (12) and of Smith (14). Similar treatment with petroleum ether is without effect.

Stoll and Wiedemann (12) have stated that electrolytes, *e.g.* NaCl, split the complex and cause the color to pass into the ether phase on shaking. It seemed desirable to re-examine this point in view of the fact that several authors report the use of NaCl for extractive purposes. Suspensions of the green material, from bean, were made 0.25 M with respect to any one of the following salts:  $\text{NH}_4\text{CNS}$ ,  $\text{KCNS}$ ,  $\text{LiCl}$ ,  $\text{KCl}$ ,  $\text{NaCl}$ , and  $\text{KI}$ . The solutions were then gently shaken with ether; none of the chlorophyll, as compared with a control, passed into the ether layer. The solutions were allowed to remain in contact with both ether and salt for 12 hours at  $0^\circ\text{C}$ . and re-examined. In most cases, there was a noticeable precipitation of the complex. Other than this, none of the chlorophyll had passed into the ether layer during the intervening time.

**Particle Size.**—Lubimenko's (5) extracts were transparent and the pigment could not be removed by centrifugation, although both Mommaerts (11) and Smith (14) state that the chloroplast material could be centrifuged at rather moderate rates of speed (3000 to 4000 R.P.M.). Shafer (13), however, noticed that his suspensions, although never clear, were difficult to centrifuge. In a more extensive investigation of this point, Stoll and Wiedemann (12), as well as Anson (15), pointed out that their preparations were optically empty in bright field, when oil-immersion was used, but, in dark field, Stoll and Wiedemann noticed Brownian motion of particles, which, they suggest, had been formed by agglomeration. We, too, have found that the purified pigment-protein, prepared as described above, contains particles or agglomerates sufficiently large for electrophoretic examination. Centrifugation for 1 hour at 3000 R.P.M. produced a preparation that was optically empty in bright field (1100  $\times$  magnification). Suspensions centrifuged for 2 hours at the same speed, although optically empty in bright field, nevertheless exhibited lively Brownian motion at the limits of microscopic visibility in dark field. It is possible that the elementary particle is large, in view of the finding of Loring, Osborn, and Wyckoff (18) that its sedimentation constant is extremely large. In contrast to this, Anson (15) found that the green particles were almost completely sedimented at 20,000 R.P.M., sedimenting more rapidly than the molecules of tobacco mosaic virus.

**Electrophoresis.**—Electrophoretic data obtained with two different instru-

ments were in excellent agreement. The mobility-pH curve of bean is shown in Fig. 1. The various circles represent similar samples prepared at different times. The green microscopic particles, which make up the suspension, are isoelectric at pH 4.70 in *M*/50 acetate buffer at 25°C. The particles migrate independent of size, shape, or degree of clumping. Particles at the limits of microscopic visibility migrated at the same rate as the larger ones. In fact, the particles behaved as though their surfaces were exceedingly uniform.

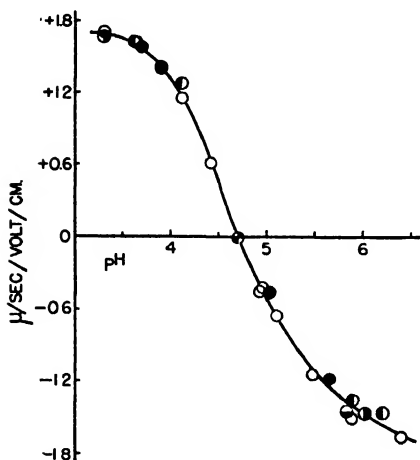


FIG. 1

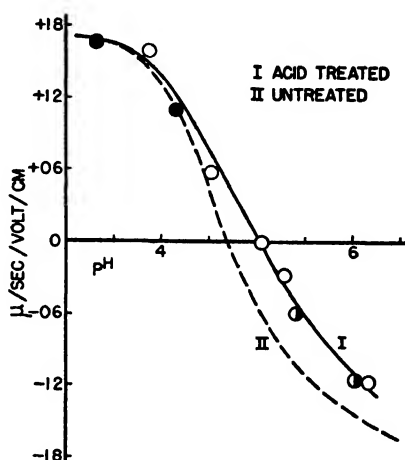


FIG. 2

FIG. 1. The electrophoretic mobility-pH curve of the chlorophyll-protein complex from navy bean in *M*/50 acetate buffer, at  $\mu = 0.02$  and 25°C. Different circles represent various preparations. The smooth curve has been drawn to fit the points. The isoelectric point is at pH 4.70.

FIG. 2. The effect of *M*/25 acetic acid on the electrophoretic mobility of the chlorophyll-protein complex from navy bean (curve I). The different circles represent various preparations treated with acid at pH 3.1 and resuspended in *M*/50 acetate buffer, at  $\mu = 0.02$  and 25°C. Curve II is a smooth (dashed) curve drawn from Fig. 1 for comparison. The isoelectric point of the acid-treated material is at pH 5.05.

Under these conditions, the curve tends to flatten out on the negative side at about pH 6.5 and on the positive side at pH 3.6.

At and below the isoelectric point the chromoprotein is readily flocculated, which confirms Smith's observation that suspensions flocculated at pH 4.5 or below have been altered by the acid. This raised the questions as to whether the flocculation is the result of denaturation and whether it is reversible. The complex was therefore treated with *M*/25 acetic acid at pH 3.1 (above the limit of pheophytin formation) for about 30 minutes. The treated material was then resuspended in more alkaline buffers and its mobility determined (Fig. 2). Curve I shows the altered mobility of the acid-treated material;

the various circles represent different preparations of the chlorophyll-protein complex. The dashed curve (II) is that of the untreated material, drawn for comparison. It is evident that the behavior of the material has been so altered that its isoelectric point now lies at pH 5.05. The curves are nearly parallel on the negative side but converge on the positive side. This suggests that nearly all the experiments on the acid side of the isoelectric point in Fig. 1 were affected by the acid conditions under which the measurements had been made. Investigations of the rate of this denaturation indicated that it is virtually complete within 5 to 10 minutes at pH 3.1 and 25°C. At pH values more basic than 3.1, the process is less rapid.

A similar experiment was done with *Aspidistra*. The mobility-pH curves for both the native and acid-treated material at an ionic strength of 0.02 and 25°C. are presented in Fig. 3. The closed circles designate the behavior of the untreated material and the open circles, the acid-treated. Notice the sharp discontinuity in the curve in the region of the isoelectric point. This again indicates that acid media can denature the complex and alter its isoelectric point. In fact, it is only by extrapolation that a value for this point can be obtained, near pH 3.9. When the complex was exposed to acidities below pH 4.24, its mobility was rapidly altered and the shifted curve on the acid side of the isoelectric point was found. If now the material was treated with  $m/25$  acetic acid at pH 3.1 and resuspended in more basic buffers, results such as those represented by the open circles were obtained. These form a smooth curve (dashed) continuous with the untreated material in acid buffers. The isoelectric point was now at pH 4.24. It is evident from Figs. 1 and 3 that the behavior of the complex is different in the two species.

Menke (10) has reported the isolation of a chloroplast substance and a cytoplasmic substance by fractional precipitation with ammonium sulfate. This suggested to us that the results obtained above might have been conditioned by the presence of impurities originating from the cytoplasm. That appreciable amounts of dissolved protein, capable of being adsorbed by the chlorophyll-protein aggregates, were not present in the highly purified preparations of the complex could be demonstrated by suspending particles of quartz or collodion in clear green suspensions of the complex obtained by centrifugation at 3000 R.P.M. It has been repeatedly shown (23) that when inert particles are suspended in protein solutions, they assume an electrophoretic mobility that is usually characteristic of the mobility of the dissolved protein molecules. Similarly, these added particles assumed an electrophoretic behavior identical, within the limits of error, with the aggregates of the complex. These results are shown by the points on Curve I of Fig. 4, which has been drawn from Fig. 1 for comparison.

There was, of course, the possibility that proteins from the cytoplasm might

be readily adsorbed, both by chlorophyll-protein and by inert surfaces. The supernatant, which remains after the first precipitation of *Phaseolus* with ammonium sulfate, still contains some of the chlorophyll-protein complex. To remove this residue, the concentration of ammonium sulfate was raised to

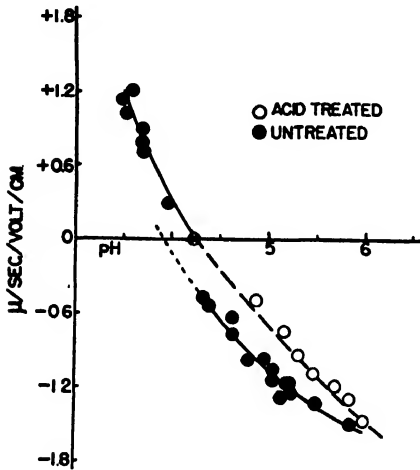


FIG. 3

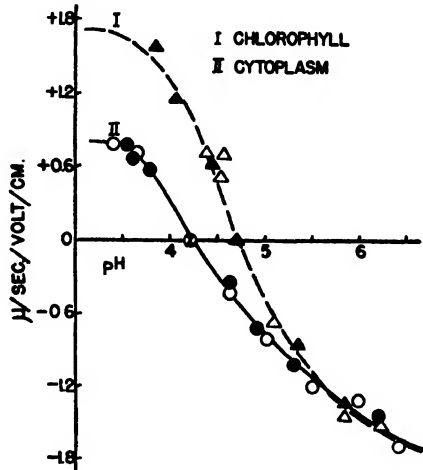


FIG. 4

FIG. 3. The electrophoretic mobility-pH curve of the chlorophyll-protein complex from *Aspidistra* in  $M/50$  acetate buffer, at  $\mu = 0.02$  and  $25^\circ\text{C}$ . The closed circles represent the behavior of the untreated material, although those in the acid region show a definite discontinuity. The open circles represent the behavior of the complex treated with  $M/25$  acetic acid at pH 3.1 and resuspended in more alkaline buffers. The acid-treated material forms a smooth curve with the positively charged particles through an isoelectric point at pH 4.24. The isoelectric point of the undenatured material can only be obtained by extrapolation. It is at pH 3.9.

FIG. 4. A comparison of the electrophoretic mobility-pH curves of quartz ( $\bullet$ ,  $\blacktriangle$ ) and collodion ( $\circ$ ,  $\triangle$ ) coated with the chlorophyll-protein complex and the cytoplasmic protein complex, in  $M/50$  acetate buffer, at  $\mu = 0.02$  and  $25^\circ\text{C}$ . The dashed curve is drawn from Fig. 1 for comparison. The smooth curve (II) has been drawn to fit the points. The isoelectric point of the adsorbed cytoplasmic protein is at pH 4.22.

half saturation, the pigment-protein complex was precipitated, and centrifuged. The yellow-brown supernatant was then dialyzed against  $M/100$  phosphate buffer at  $0^\circ\text{C}$ . Quartz and collodion particles were suspended in the clear dialyzed supernatant and their electrophoretic mobilities determined. The data are plotted as Curve II of Fig. 4. The isoelectric point of the cytoplasmic protein adsorbed by the particles is at pH 4.22, as compared with pH 4.70 for the chromoprotein.

## DISCUSSION

A comparison of the data for the monocotyledonous *Aspidistra* and the dicotyledonous *Phaseolus* shows that distinct differences exist between genera. Although the analyses of Smith (14) suggested to him that *Spinacia* and *Aspidistra* were so nearly identical that analyses could be averaged, he does, however, report a difference in their response to precipitating salts. Not only do *Aspidistra* and navy bean differ in their ease of precipitation by ammonium sulfate, the former precipitating like a globulin and the latter like an albumin, but their mobility-pH curves (diagrammed in Figs. 3 and 4) are clearly distinct. At the same conditions of ionic strength and temperature, the navy bean has an isoelectric point at pH 4.70 and *Aspidistra* (when extrapolated) at about pH 3.9. These differences between the genera indicate a very definite specificity in the constitution of the complex, despite the fact that the chlorophyll is the same in both. This difference may be attributed to variations in either the lipid or the protein bearer.

The effect of various uni-univalent electrolytes on the stability of the chlorophyll-protein complex seems to contradict the results of Stoll and Wiedemann (12). They claimed that when a suspension of the green material, treated with an electrolyte like NaCl, was shaken with ether, the pigment readily passed into the ether layer, and concluded that this was due to the presence of the salt. We find, however, that this results only when the suspension is vigorously shaken with ether or when the complex is allowed to warm up to room temperature; both of these treatments, we have shown, however, split the chromoprotein in the absence of salt. These conclusions agree with those of Shafer (13) and Anson (15), who have used dilute salt solutions to prepare stable suspensions of the green material.

The mobility-pH curves of *Phaseolus* and *Aspidistra*, together with their isoelectric points, fall within the range characteristic of protein systems. The analytical evidence of Mommaerts, Stoll and Wiedemann, and Smith also indicates that the complex contains a protein. This, together with the facts that the green material is thrown out of solution by protein precipitants and sediments as a large unit in the centrifugal field (18), indicates quite definitely that chlorophyll is bound to a protein.

From Fig. 1 it would appear that the protein from bean remains essentially unchanged over the entire range of pH investigated. Previous reports (14) had indicated, however, that the pigment-protein complex flocculates and is irreversibly denatured below the isoelectric point. Not only did we find this, but when the green material, which had been treated with acid below the isoelectric point, was brought back into more alkaline buffers, its electrophoretic mobility no longer agreed with that of the native, undenatured material but was shifted to more basic values. In the case of *Aspidistra*, the differences between the native and the acid denatured material were more

marked. In fact, with the techniques employed here, it was impossible to obtain an isoelectric point for the undenatured material. In this respect, the chlorophyll-protein complex is similar to hog thyroglobulin, which, as shown by Heidelberger and Pedersen (24), is also denatured by dilute acids and exhibits a shifted mobility curve below its isoelectric point.

The preparations used in this investigation were apparently homogeneous and the data were remarkably uniform. Neither the aggregates of green material nor quartz and collodion particles coated with it exhibited any electrophoretic differences within the limits of experimental error. Even if the aggregates are not agglomerates of the chlorophyll-protein complex, at least they are coated by the same dissolved or dispersed substance that is adsorbed by quartz or collodion. In any case, it is clear that the protein adsorbed from the cytoplasmic material by quartz or collodion is quite different from that forming the surface of the green particle (Fig. 4). This does not imply that only one protein is present in the cytoplasm but, as in the case of other mixed systems (25), it seems that one, at least, tends to be selectively adsorbed. In the light of the foregoing evidence, it seems most likely that what we have been investigating are the surface properties of the chlorophyll-protein complex itself. The moving boundary method of electrophoresis should clarify the situation.

#### SUMMARY

1. Reported effects of different conditions on the stability of the purified chlorophyll-protein complex have been confirmed.

2. The electrophoretic behavior of the chlorophyll-protein complex prepared from two unrelated species of plants (*Aspidistra elatior* and *Phaseolus vulgaris*) has been investigated and shown to be dissimilar. In M/50 acetate buffer at 25°C, the isoelectric point of the complex from *Phaseolus* is at pH 4.70, whereas that from *Aspidistra* is at pH 3.9 (extrapolated). These values fall within the usual range of protein isoelectric points.

3. Treatment with weak acids causes an irreversible denaturation of the protein complex from both species, with a resultant shift in the mobility-pH curves to more basic values.

4. Differences in electrophoretic behavior between the chlorophyll-protein complex and the cytoplasmic proteins of *Phaseolus* have been demonstrated. The isoelectric point of the latter is at pH 4.22.

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# ELECTRIC IMPEDANCE OF THE FROG EGG

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## INTRODUCTION

The analysis of measurements of the alternating current impedance of tissues, cell suspensions, and single cells has shown that the cell membrane has a capacity of about  $1 \mu\text{f}/\text{cm}^2$  and an associated dielectric loss similar to that found in solid dielectrics. Because the capacity is relatively constant for many cells and under various conditions, it and the loss are probably characteristics of the molecular structure in the ion-impermeable part of the membrane. In the case of a few cells it has been possible to measure a leakage resistance of the membrane. This resistance is dependent upon the condition of the cell and may be ascribed to the transfer of ions across the membrane. The ion permeability of the membrane is an important factor in the functioning of the cell but its electrical measurement as a leakage conductance is dependent upon a favorable combination of the size and shape of the cell and the resistance of the medium (Cole, 1937, 1940). The ideal material for physiological work of this nature is a single large cell of regular shape which is normally found in an environment of low salt concentration. The frog egg seemed to meet these requirements and this work was undertaken to determine the plasma membrane resistance of the unfertilized and fertilized egg. In addition it has been possible to determine the membrane capacity and phase angle, and the internal resistance for the unfertilized and fertilized egg in spring water and in diluted amphibian Ringer's. The stability of the impedance was also of interest because of the spontaneous, rhythmic impedance fluctuations observed in the trout egg by Hubbard and Rothschild (1939).

## Material and Apparatus

Single eggs of the leopard frog, *Rana pipiens*, were used throughout. The eggs were stripped from the frog and immediately placed in the measuring cell. Eggs which were to be fertilized were stripped from females previously injected with frog pituitaries (Rugh, 1934) so that they were sexually mature in the fall and winter months. These eggs were allowed to fall into spring water or 10 per cent Ringer's solution (1 part amphibian Ringer's to 9 parts of distilled water), artificially inseminated, and placed in the measuring cell. Only those eggs, eight in number, which showed subsequent development to at least the two-cell stage were designated as fertilized eggs. Checking the fertilizability of the unfertilized eggs (two in number) after a run would



not have had any particular value because it is well known that these eggs lose their fertilizability very soon after removal from the body.

In addition to the fertilized and unfertilized group, there was another group of eggs designated "undetermined" (nine eggs). These eggs were treated with sperm solution prior to measurement but whether or not their insemination was successful could not be proved, inasmuch as when it was next possible to examine them some days later, they had undergone decomposition. Thus it was impossible to determine whether they had died because of injury subsequent to fertilization and early development or whether they had been unsuccessfully inseminated and never developed at all.

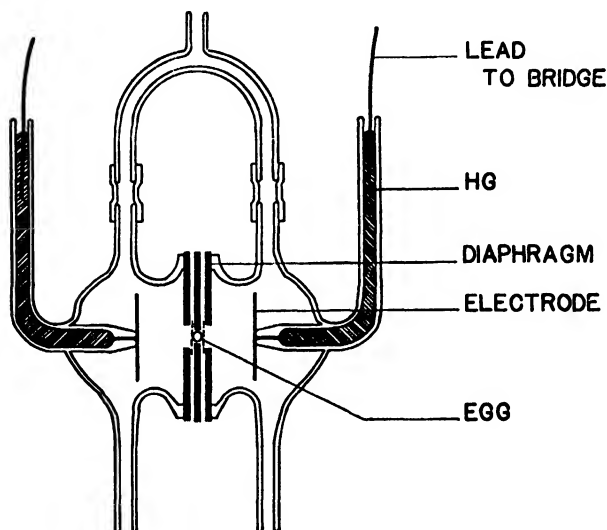


FIG. 1. Chamber for measuring impedance of single frog eggs. When assembled the diaphragms are in contact. (The broken lines on either side of the egg represent pieces of cheesecloth which hold the egg in place.)

A few measurements were made upon eggs which were known to be dead. In these cases the impedance was independent of frequency and close to that obtained for the medium alone. Measurements were also made on the vitelline membrane alone which showed that it had approximately the conductivity of the medium.

After preliminary experiments with other types, a measuring cell of the form used by Frick and Morse (1925) was adopted for these experiments. As is shown in Fig. 1, each platinized platinum electrode of this cell was sealed into a cup with a ground lip. When the two cups were clamped together directly or with flat diaphragms between them a watertight joint was formed. The egg was placed in a circular hole, 0.216 cm. in diameter, drilled in the center of a hard rubber diaphragm, 0.164 cm. thick. The adhering jelly served to center the egg in the hole and a small piece of moistened cheesecloth on each side kept the egg from being forced out of the hole by momentary pressure differences. Each piece of cheesecloth in turn was held in

place by another diaphragm having a center hole, 0.6 cm. in diameter. These three diaphragms were placed between the ground lips of the electrode cups, the whole clamped together and filled with spring water or 10 per cent amphibian Ringer's solution through the tubes at the top and bottom of each cup.

An alternating current Wheatstone bridge similar to that described by Cole and Curtis (1937) was used for the measurements. A set of measurements was made at eight frequencies from 50 cycles to 10 kc. first with and then without an egg in place. At each frequency the parallel resistance,  $R_p$ , and capacity,  $C_p$ , were measured. The temperature was not controlled but several check measurements were taken, usually at 1 kc., during the course of each run and correction made for the effect of temperature change when necessary.

### Corrections and Calculations

In other work it has usually been necessary to apply a correction for the polarization impedance of the electrodes but in this case with electrodes 3.3

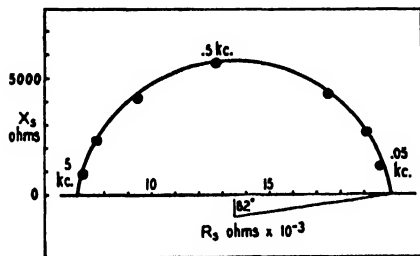


FIG. 2 a

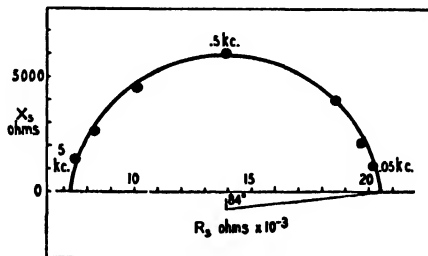


FIG. 2 b

FIG. 2. Frequency impedance locus, *i.e.* series resistance,  $R_s$ , vs. series reactance,  $X_s$ , of (a) unfertilized and (b) fertilized frog egg. Frequencies indicated are in kilocycles per second.

cm. in diameter this impedance was negligible. The series resistance,  $R_s$ , and series reactance,  $X_s$ , at each frequency were then computed from the measured resistance and capacity and plotted as abscissae and ordinates, respectively, of the impedance locus (Cole, 1928 a). A typical locus for an unfertilized frog egg is shown in Fig. 2 a and for a fertilized egg in Fig. 2 b.

### End Effect

Before these data can be interpreted, it is necessary to consider the current flow between each electrode and the end of the cylindrical hole in the diaphragm facing it. This resistance of this region depends upon the specific resistance of the electrolyte, the separation between the electrode and the diaphragm, and the diameters of the hole, the vessel, and the electrodes. The current from the electrode converges rapidly in the electrolyte outside the diaphragm as it approaches the hole and encounters a resistance which is practically independent of the length and contents of the hole. This resistance could be

measured directly if the central hole were filled with metal or if a very thin diaphragm having a central hole of the same diameter were used. However, these measurements would be complicated by the polarization impedance of the metal faces in the first case and the dielectric capacity of the diaphragm in the second case. A third possibility is to vary the length of the hole by using diaphragms of different thickness and obtain the resistance for zero length by extrapolation. The results of this latter experiment with five diaphragms having holes 0.216 cm. in diameter are shown in Fig. 3. The measured resistance with the diaphragm 0.164 cm. thick used for the eggs is 2,600 ohms and the extrapolated resistance for zero thickness is 1,300 ohms. Consequently the end effect is 50 per cent of the resistance observed for the experimental

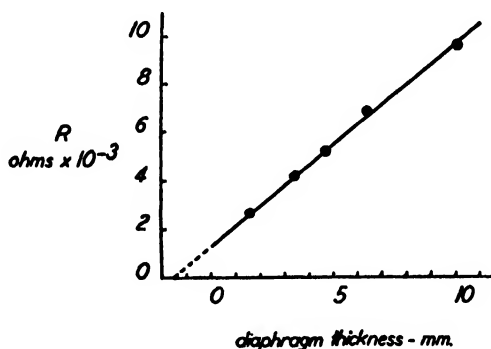


FIG. 3. The overall resistance,  $R$ , in thousands of ohms, of measuring chamber and diaphragm plotted against diaphragm thickness in millimeters. Each diaphragm had a central hole 0.216 cm. in diameter. The medium was spring water.

diaphragm without an egg in place. This value was then subtracted from the series resistance obtained at each frequency to correct for the resistance of the electrolyte on each side of the diaphragm.

From the extrapolation to the axis of abscissae, it is found that the end effect is equivalent to an added hole length of 0.16 cm. If the equivalent hole length  $l'$  is  $l' = l + \gamma a$ , where  $l$  is the actual length and  $a$  the radius of the hole, then  $\gamma = 1.48$  experimentally. An approximate calculation for extensive electrolyte and large distant electrodes gives  $\gamma = 1.57$  and Rayleigh gives  $\gamma = 1.64$  as a better value (Jeans, 1927, page 358). However, the electrodes are only 0.75 cm. from each side of the disc and this reduces the computed value of  $\gamma$  by about 0.10 (Gray, Mathews, and MacRobert, 1922, page 145). The finite diameter of the cups and the electrodes increases  $\gamma$  but only by a negligible amount (Gray, Mathews, and MacRobert, 1922, page 147). The theoretical value is then in the neighborhood of  $\gamma = 1.5$  and the agreement with the experimental value of  $\gamma = 1.48$  is better than should be expected.

*Effective Volume Concentration*

The next step in the analysis is to consider the distribution of current flow around and through the egg in the cylindrical hole. The resistance,  $r$ , of a cubical cell containing a small sphere is most easily obtained by the assumption that the field strength at the boundaries of the cell is not modified by the sphere. On this basis Fricke (1924) found

$$\frac{1 - r_1/r}{3} = \rho \frac{1 - r_1/r_2}{2 + r_1/r_2}$$

where  $r_1$  and  $r_2$  are the specific resistance of the medium and the sphere respectively, and  $\rho$  is the fraction of the volume occupied by the sphere. Obviously this equation will apply for any shape of boundary if it is everywhere sufficiently remote from the sphere. It will also apply for a dilute suspension of spheres in which the field at a single sphere is not affected by the presence of the others. As the fractional volume  $\rho$  is increased, the field inside a sphere is altered by the walls or by the other spheres of a suspension. If this internal field remains uniform and is determined by the average field in the system, the next approximation is the Maxwell (1873, section 313) equation for a random suspension

$$\frac{1 - r_1/r}{2 + r_1/r} = \rho \frac{1 - r_1/r_2}{2 + r_1/r_2} \quad (1)$$

This equation also applies to an approximately cubical arrangement of spheres (Rayleigh, 1892) and consequently to a single sphere in a nearly cubical cell. We then expect that the exact shape of the measuring cell is not particularly important and that equation (1) will also apply to a sphere in a short cylindrical hole. This is easily shown to be true if the internal field is uniform and determined by the average field in the system. However, when the effect of the sphere becomes too large, this assumption will not be valid and a more complete theory is necessary to give these limits. Since this theory is not available the validity of equation (1) can only be established experimentally.

In the case of a non-conducting sphere, which is the most critical theoretically and the most easily tested, equation (1) becomes

$$(1 - r_1/r)/(2 + r_1/r) = \rho/2 \quad (2)$$

To test this equation for a non-conducting sphere, one end of a fine glass rod was melted down until it became a sphere of the proper size. This sphere was then held in the center of the hole by the attached rod which in turn was cemented to a glass bridge on one side of the diaphragm. The effect of the rod and bridge was negligible because they were small and in regions of low current density. In Fig. 4, the left hand side of equation (2) is computed from the

measured resistances and plotted against the volume,  $v$ , of the sphere. The linear relation for small values of  $v$  justifies the use of equation (2).

The linear relation between these two quantities is expected according to equation (2) for small values of  $v$ . With the larger values of  $v$ , the assumptions of equation (2) are no longer valid, and the measurements do not lie on the straight line. For example, if the diameter of the sphere is equal to that of the cylindrical hole the resistance is infinite and  $(1 - r_1/r)/(2 + r_1/r) = 0.5$ . But according to equation (2), the resistance should not become infinite until  $v = V$ , the volume of the cylinder. Then by extrapolation of the straight line obtained for small  $v$  we find  $V = 6.3 \text{ mm}^3$ . Although the actual volume of the cylinder was  $6.0 \text{ mm}^3$ , the extrapolated value may be used as an apparent

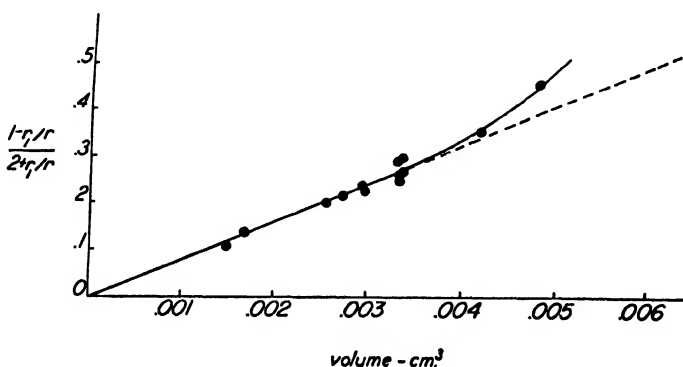


FIG. 4. The volume of the glass sphere in  $\text{cm}^3$ , plotted against the expression  $(1 - r_1/r)/(2 + r_1/r)$ , where  $r_1$  is the resistance in ohms of the medium and  $r$ , the resistance in ohms with the sphere in place. The experimental points are joined by an unbroken line, while the extrapolation of the straight line is represented by a broken line.

or effective volume. Equation (2) may then be used for values of  $\rho$  up to 50 per cent and  $\rho = v/V$ , where  $v$  is the volume of the egg computed from its diameter and  $V$  is the effective volume of the cylindrical cell.

#### Internal Resistance

The impedance loci of Fig. 2 are approximately circular arcs and may be extrapolated to the resistance axis to give  $r_0$ , the zero frequency resistance at one end and  $r_\infty$ , the infinite frequency resistance at the other end. If we assume that the membrane impedance is negligible at infinite frequency, the internal resistance  $r_2$  may be computed by equation (1) where  $r = r_\infty$

$$\frac{1 - r_1/r_\infty}{2 + r_1/r_\infty} = \rho \frac{1 - r_1/r_2}{2 + r_1/r_2} \quad (3)$$

The values for  $r_2$  so obtained are given in Table I.

*Membrane Resistance*

In a similar manner the resistance,  $\bar{r}_2$ , of an equivalent uniform sphere at zero frequency may be computed when  $r = r_0$ ,

$$\frac{1 - r_1/r_0}{2 + r_1/r_0} = \rho \frac{1 - r_1/r_2}{2 + r_1/r_2}. \quad (4)$$

If the egg has an electrically homogenous interior of specific resistance  $r_2$  surrounded by a thin membrane of resistance  $r_3$  per unit area, the equivalent resistance is given by

$$\bar{r}_2 = r_2 + r_3/a \quad (5)$$

where  $a$  is the radius of the egg (Cole, 1928 *a*). The membrane resistances may now be obtained from the values of  $\bar{r}_2$ ,  $r_2$ , and  $a$  previously found. These results are given in Table I.

*Membrane Phase Angle and Capacity*

The formulae previously used to calculate the membrane capacity cannot be used because they have been based on the assumption that the membrane conductance was negligible. The fact that the zero frequency resistances were usually larger for the medium alone than with an egg in place shows that the equivalent resistance of the egg is less than the resistance of the medium and that the assumption of a negligible conductance is not valid.

On the basis of previous work, summarized by Cole (1940), it will be assumed that the membrane resistance,  $r_3$ , and the dielectric impedance,  $z_4$ , are in parallel. The membrane impedance  $z_m$  is then given by  $1/z_m = 1/r_3 + 1/z_4$ . The equivalent egg resistance,  $\bar{r}_2$ , in equation (1) is now replaced by the equivalent impedance  $\bar{z}_2 = r_2 + z_m/a$  and by rearrangement equation (1) becomes

$$z = r_1 \frac{(1 - \rho)r_1 + (2 + \rho)r_2 + [(1 - \rho)r_1 + (2 + \rho)(r_2 + r_3/a)]z_4/r_2}{(1 + 2\rho)r_1 + 2(1 - \rho)r_2 + [(1 + 2\rho)r_1 + 2(1 - \rho)(r_2 + r_3/a)]z_4/r_2} \quad (6)$$

This equation is of the same form as for a negligible membrane conductance. For a constant phase angle dielectric impedance  $z_4 = |z|(j\omega)^{-\alpha}$  the impedance locus of  $z$  should be a circular arc of central angle,  $2\varphi = \alpha\pi$  (Cole, 1928 *a*, 1932). As seen in Fig. 2 this is approximately true. The values of  $\varphi$  found are between  $80^\circ$  and  $88.5^\circ$  and the average value is  $86^\circ$ .

Solving equation (6) for  $z_4$  and introducing the values for  $r_0$  and  $r_\infty$  from equations (3) and (4), we obtain

$$z_4 = \frac{[(1 + 2\rho)r_1 + 2(1 - \rho)r_2]r_3}{(1 + 2\rho)r_1 + 2(1 - \rho)(r_2 + r_3/a)} \cdot \frac{z - r_\infty}{r_0 - z} \quad (7)$$

The frequency for which the reactance component of the measured impedance  $z$  is a maximum may be called the characteristic frequency,  $\tilde{\nu}$ , (Cole and Curtis, 1936). At this frequency, the absolute value

$$\left| \frac{z - r_{\infty}}{r_0 - z} \right| = 1$$

and if  $z_4$  were that of a pure capacity  $z_4 = 1/c_m \omega$  we have

$$c_m = \frac{1}{\tilde{\omega} \left( r_2 + \frac{1 + 2\rho}{2(1 - \rho)} r_1 \right)} + \frac{1}{\tilde{\omega} r_2} \quad (8)$$

where  $\tilde{\omega} = 2\pi\tilde{\nu}$ .

This equation is not strictly valid because the phase angle of  $z_4$  is somewhat less than  $90^\circ$ , but it gives the capacity component of the membrane impedance to within 1 per cent in the present experiments. The values of  $c_m$  obtained under various conditions are given in Table I.

#### DISCUSSION OF RESULTS

The results of the study of the electrical parameters of the frog egg are summarized in Table I.

**Internal Specific Resistance.**—The internal specific resistance of the cell,  $r_2$ , is dependent upon the concentration and mobilities of ions in the cell interior. It was calculated on the assumption that the membrane has a negligible resistance at the infinite frequency extrapolation. The average value obtained for the nineteen eggs studied was 570 ohm cm. This value can be compared with the value of 200 ohm cm. obtained by Holzer (1933) for unfertilized trout eggs, which are also fresh water eggs and with the values obtained by Cole *et al.* for various marine eggs (*Arbacia* (1928 b, 1936 b, 1938), *Asterias*, (1936 a), *Hippoonö* (1935), *Cumingia* and *Chaetopterus* (1938)) which also are approximately 200 ohm cm.

**Membrane Resistance.**—The membrane resistance is probably a measure of the membrane permeability to ions. It is dependent upon the condition of the cell, being altered during cell activity (Cole and Curtis, *Nitella* (1938), squid axon (1939)) and during narcosis (Guttman, 1939). The average membrane resistance value obtained for the frog egg was 170 ohm cm<sup>2</sup>. Because of technical difficulties it has in the past been impossible to measure the membrane resistance of most cells studied with impedance methods. There are therefore few data with which the frog egg value may be compared. Holzer (1933) obtained a value of 6000 ohm cm<sup>2</sup>. for the membrane resistance of unfertilized trout eggs. The value obtained for the frog egg lies in the general range of resistances found by Blinks for *Valonia* (1930) and *Halicystis* (1938), by Cole and Hodgkin for the squid axon (1939), and by Bozler and Cole (1935)

for frog sartorius muscle. This suggests that the ion permeabilities of the membranes of these cells are comparable.

**Membrane Capacity.**—Unlike membrane resistance, membrane capacity is a fairly inert characteristic of the cell membrane, changing neither during activity nor narcosis of the cell. It probably is associated with the ion-impermeable aspect of the cell membrane. The average value obtained for the frog egg was  $2.0 \mu\text{f}/\text{cm}^2$ . Almost all living cells thus far measured have

TABLE I

*Calculation of Electrical Parameters of Single Frog Eggs under Various Conditions*

$r_2$ , internal specific resistance in ohm cm.;  $r_3$ , membrane resistance in ohm  $\text{cm}^2$ ;  $c_m$ , membrane capacity in  $\mu\text{f}/\text{cm}^2$ . Numbers in parentheses indicate number of eggs in each case.

$r_2$	10 per cent Ringer	Spring water	Average
Fertilized . . . . .	625 (6)	510 (2)	620 (8)
Unfertilized . . . . .	480 (1)	580 (1)	540 (2)
Undetermined . . . . .	590 (8)	320 (1)	560 (9)
Average . . . . .	600 (15)	430 (4)	570 (19)
$r_3$	10 per cent Ringer	Spring water	Average
Fertilized . . . . .	140 (6)	390 (2)	200 (8)
Unfertilized . . . . .	70 (1)	430 (1)	225 (2)
Undetermined . . . . .	120 (8)	190 (1)	130 (9)
Average . . . . .	120 (15)	350 (4)	170 (19)
$c_m$	10 per cent Ringer	Spring water	Average
Fertilized . . . . .	1.8 (6)	1.4 (2)	1.7 (8)
Unfertilized . . . . .	2.7 (1)	1.7 (1)	2.2 (2)
Undetermined . . . . .	2.3 (8)	2.2 (1)	2.3 (9)
Average . . . . .	2.1 (15)	1.7 (4)	2.0 (19)

surprisingly constant membrane capacities of about 1 or  $2 \mu\text{f}/\text{cm}^2$ . Into this general scheme the membrane capacity of the frog egg fits rather well.

**Effect of Alteration of External Medium.**—Since frog eggs develop equally well in spring water and in a 10 per cent amphibian Ringer solution, it was possible to study the effect of the external medium upon the cell parameters by making observations on eggs in each type of medium and then comparing the results.

It is of interest that the membrane capacity,  $c_m$ , which is, as we have seen, in general a rather inert characteristic of the cell, is not significantly different when the egg is in 10 per cent Ringer ( $2.1 \mu\text{f}/\text{cm}^2$ ) from the value when the egg is in spring water ( $1.7 \mu\text{f}/\text{cm}^2$ ) (Table I). On the other hand, the membrane resistance,  $r_3$ , is definitely higher in the case of the spring water values ( $350 \text{ ohm cm}^2$ ) than in the 10 per cent Ringer values ( $120 \text{ ohm cm}^2$ ). That



the membrane resistance should change is not very surprising since this value probably represents an equilibrium with both the external and internal media. The internal specific resistance,  $r_2$ , may also be dependent upon the external medium, being 600 ohm cm. when the eggs were in 10 per cent Ringer and 430 ohm cm. when the eggs were in spring water. If this difference is significant it indicates an accumulation of ions in the cell interior from the more dilute medium.

*Effect of Fertilization.*—Fertilization of the eggs had little effect upon the cell parameters investigated (Table I). Of the nineteen eggs studied, eight were successfully inseminated just before measurement, as was shown by their subsequent normal development. These are designated "fertilized" in Table I. Two eggs were unfertilized and are so designated. Nine eggs whose fertilization was uncertain are listed as "undetermined" in Table I.

The data obtained for the "undetermined" group of eggs do not fall into two distinct classes. This means that either (1) these eggs were all fertilized eggs or all unfertilized eggs or (2) the group includes both fertilized and unfertilized eggs, and the membrane resistance, membrane capacity, and specific resistance of the egg interior do not change upon fertilization. While it is possible that the nine eggs were either all fertilized or all unfertilized, the second assumption is more probable. In any case, there is no marked difference in membrane resistance, membrane capacity, and specific resistance of the egg interior in those eggs definitely known to be fertilized or unfertilized.

McClendon (1910) and later Gray (1916) demonstrated a decrease in the resistance of centrifuged echinoderm eggs on fertilization. On the other hand no marked change in membrane resistance was found after fertilization in *Arbacia* eggs (Cole and Spencer (1938)), or in *Hippoonö* eggs (Cole, 1935). Both the *Arbacia* and the *Hippoonö* membrane capacities increased on fertilization, unlike the frog egg. No such change in membrane capacity on fertilization occurs in *Cumingia* or in *Chaetopterus* eggs (Curtis and Cole, 1938).

*No Spontaneous Rhythmical Impedance Changes in Frog Egg.*—Although Hubbard and Rothschild (1939) have reported small spontaneous rhythmical impedance changes in the unfertilized and fertilized trout egg after soaking for 12 hours in running tap water, no similar phenomenon was observed in the frog egg.

We wish to thank Prof. Lester Barth of the Department of Zoology, Columbia University, and his assistants, Drs. Edgar Zwilling and John Moore for the pituitary-injected frogs which were used in these experiments.

#### SUMMARY

Electrical impedance measurements were made upon unfertilized and fertilized eggs of the leopard frog, *Rana pipiens*, over a frequency range of

0.05 to 10 kc. Average values of 170 ohm cm.<sup>2</sup> were obtained for the plasma membrane resistance of the egg, 2.0  $\mu$ f/cm.<sup>2</sup> for the plasma membrane capacity, 86° for the phase angle of the membrane, and 570 ohm cm. for the specific resistance of the interior. These values did not change upon fertilization. No spontaneous rhythmical impedance changes such as have been found by Hubbard and Rothschild in the trout egg were found in frog eggs.

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# PANTOTHENIC ACID AND THE UTILIZATION OF GLUCOSE BY LIVING AND CELL-FREE SYSTEMS\*

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## I

### INTRODUCTION

Since the discovery of the vitamin properties of pantothenic acid much interest has been aroused as to its biochemical function. Williams and co-workers found that it stimulated the deposition of carbohydrate by green plants without affecting their nitrogenous constituents.<sup>1</sup> The glycogen storage of yeast was also found to be increased<sup>2</sup> by incubating it with pantothenic acid. Yeast grown on a medium deficient in pantothenic acid possessed a low fermenting ability which was greatly increased by the addition of pantothenic acid.<sup>3</sup> Such yeast was found to have a much lower pantothenic acid content than normal yeast. The rate of fermentation of sucrose by dialyzed yeast maceration juice was also observed to be slightly accelerated by the addition of calcium pantothenate. These experiments indicated that pantothenic acid was involved, probably as a coenzyme, in some stage of carbohydrate metabolism.

In the present study these experiments were repeated and extended. Since the tissues of chicks grown on a diet deficient in pantothenic acid are low in pantothenic acid,<sup>4</sup> deficient brain and muscle served as basal tissues on which the effect of added pantothenic acid could be studied. Maceration juice preparations from yeast served as cell-free material on which similar studies could be made. All of the respiration experiments were carried out in a Warburg-Barcroft microrespirometer.

## II

### *Effect on Maceration Juice*

Maceration juice was prepared from Fleischmann's baker's yeast. This

\* The authors wish to express their appreciation to Drs. C. M. Lyman and E. E. Snell for their assistance and suggestions during the course of this work.

<sup>1</sup> McBurney, C. H., Bollen, W. B., and Williams, R. J., 1935, *Proc. Nat. Acad. Sc.*, **21**, 301.

<sup>2</sup> Williams, R. J., Mosher, Wm. A., and Rohrmann, E., 1936, *Biochem. J.*, London, **30**, 2036.

<sup>3</sup> Pratt, E. F. and Williams, R. J., 1939, *J. Gen. Physiol.*, **22**, 637.

<sup>4</sup> Snell, E. E., Pennington, D., and Williams, R. J., 1940, *J. Biol. Chem.*, **133**, 559.

juice was freed of its major portion of pantothenic acid in one of two ways: (1) by dialysis, (2) by precipitating the active enzymes with acetone and redissolving the precipitate in 1 per cent sodium chloride solution. Either of these procedures gives a preparation containing considerably less pantothenic acid than normal maceration juice as determined by the method of Pennington, Snell, and Williams.<sup>5</sup> However, it was observed that additional pantothenic acid could be liberated by enzymatic action. One such juice, on which the acetone precipitation and re-solution in NaCl solution was repeated twice, contained only 0.45  $\gamma$  per cc. of "free" pantothenic acid. Enzymatic action liberated from this juice an additional 5-6  $\gamma$  per cc. of "bound" pantothenic acid. The determination of bound pantothenic acid was carried out by incubating a portion of the juice with homogenized fresh muscle tissue and allowing the autolytic enzymes of the muscle tissue to act on the yeast juice. The difference between the pantothenic acid content of this mixture and of the fresh muscle tissue itself was taken as the sum of the "free" and "bound" pantothenic acid in the yeast juice.

TABLE I  
*Fermentation of Glucose by Dialyzed Maceration Juice*

Calcium pantothenate. ....	0	0	0	0.1 $\gamma$	0.1 $\gamma$	0.1 $\gamma$	10 $\gamma$	10 $\gamma$
CO <sub>2</sub> per hr., mm. <sup>3</sup> .....	368	379	375	356	358	374	351	379

A number of experiments were performed to determine the effect of pantothenic acid on the fermentation of glucose by the dialyzed and acetone-precipitated maceration juice. No effect due to pantothenic acid was observed. Table I shows a typical run with juice dialyzed 8½ hours at 0-10° C. against 0.9 per cent NaCl solution. A small amount of hexose diphosphate was used in each flask to convert the adenylic acid into adenosine triphosphate. No appreciable fermentation took place unless this was added.

Each flask contained 1 cc. of 0.4 M glucose, 1 cc. of KH<sub>2</sub>PO<sub>4</sub> (8 gm. per liter), 10  $\gamma$  of adenylic acid, 10  $\gamma$  of coenzyme I, 0.1 cc. of salt solution (0.025 M MgSO<sub>4</sub>, 0.05 M MnSO<sub>4</sub>, and 0.3 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), and 7 mg. of sodium hexose diphosphate. Total volume, 4 cc.

Although it is generally accepted that no coenzyme, other than cocarboxylase, is required for the decarboxylation of pyruvic acid, several experiments were carried out to test the possibility of pantothenic acid's having some effect on this stage of the fermentation reaction. Pantothenic acid showed no effect on the decarboxylation of pyruvic acid by acetone-precipitated maceration juice.

The effect of pantothenic acid on the rate of phosphorylation of glucose by

<sup>5</sup> Pennington, D., Snell, E. E., and Williams, R. J., 1940, *J. Biol. Chem.*, **135**, 213.

dialyzed maceration juice was determined. The calcium salt of pantothenic acid was used, and controls containing calcium chloride were included since calcium ion can to some extent replace magnesium in the phosphorylation enzyme. Inorganic phosphate was determined according to the method of Briggs<sup>6</sup> with the Evelyn photoelectric colorimeter. The decrease in inorganic phosphate was assumed to be the amount esterified with glucose.

The results of a typical experiment are given in Table II. The quantities shown are inorganic phosphate, and the difference between the initial reading and the test reading is the decrease in inorganic phosphate. It can be seen from the table that no effect of calcium pantothenate was observed which was not attributable to the calcium ion.

The results of these experiments with maceration juice indicate that panto-

TABLE II  
*Phosphorylation of Glucose by Dialyzed Maceration Juice*

Calcium pantothenate added	Initial	10 min.	30 min.	60 min.	180 min.
0	1.16	1.12	0.89	0.56	0.48
1 $\gamma$	1.16	1.09	0.92	0.56	0.47
10 $\gamma$	1.16	1.12	—	0.56	0.48
100 $\gamma$	1.16	1.10	0.92	0.57	0.50
15 $\gamma$ CaCl <sub>2</sub>	1.16	1.10	0.91	0.57	0.50

Figures represent milligrams of phosphorus present as inorganic phosphate.

thenic acid does not function as a dissociable coenzyme for the enzyme systems involved in the fermentation of glucose.

### III

#### *Effect on Live Yeast*

The large effect of pantothenic acid upon the fermentation of glucose by yeast deficient in pantothenic acid is apparently in contradiction to the fact that pantothenic acid showed no effect on the fermentation by maceration juice from which most of the pantothenic acid had been removed. This apparent contradiction suggested that pantothenic acid may act only in a combined form. If this is the case, one would expect the accelerating action of the pantothenic acid on the deficient yeast to be accompanied by a parallel binding of the pantothenic acid in the yeast. The following experiment was performed to determine if this binding takes place.

<sup>6</sup> Briggs, A. P., 1924, *J. Biol. Chem.*, **59**, 255.

Gebrüder-Mayer yeast was grown from a heavy seeding in a medium containing sucrose, asparagine, salts, thiamin, nicotinic acid, riboflavin, biotin, and inositol, but no pantothenic acid. A twelvefold increase was obtained. These deficient cells were centrifuged off and washed with  $\text{KH}_2\text{PO}_4$  solution before being used. A preliminary experiment using 2 mg. quantities of this yeast showed that an appreciable increase in  $\text{CO}_2$  evolution occurred on the addition of as little as 0.1 $\gamma$  of pantothenic acid.

For the determination of the pantothenic acid bound by the deficient yeast 50 mg. quantities of yeast were used. These were incubated for 4 hours at 30°C. with 5 cc. of  $\text{KH}_2\text{PO}_4$  solution (4 mg. per liter), 5 cc. of 0.4 M glucose solution, and 1 cc. of the indicated amount of pantothenic acid. The cells were then centrifuged down, washed three times with 1 per cent NaCl solution, and finally suspended in 10 cc. of acetic acid-sodium acetate buffer solution (pH = 4.5). One half of each sample was steamed for 10 minutes, then autoclaved for 15 minutes at 15 pounds pressure. The hot water extract was used for the determination of the free pantothenic acid in the

TABLE III  
*Pantothenic Acid Bound by Deficient Yeast*

Pantothenic acid added	Free pantothenic acid in 50 mg. of deficient yeast	Pantothenic acid bound by 50 mg. of deficient yeast
0	0.08 $\gamma$	0*
0.5 $\gamma$	0.06 $\gamma$	0.30 $\gamma$
5 $\gamma$	0.28 $\gamma$	0.68 $\gamma$
50 $\gamma$	0.30 $\gamma$	0.74 $\gamma$

\* Too small to determine.

cells. The other half of each sample was incubated for 36 hours at 37°C. with a mixture of clarase and caroid. This enzyme treatment<sup>7</sup> has been standardized in this laboratory for the liberation of pantothenic acid bound in tissues. This portion was used for the determination of the total pantothenic acid in the cells. The difference between these quantities was then taken as the amount of "bound" pantothenic acid.

The results of this experiment are given in Table III. It is evident from these data that the increase in the rate of fermentation by the deficient yeast is accompanied by a parallel "binding" of the added pantothenic acid in the cell, probably to form an integral part of some enzyme. Since the medium used contained no nitrogen from which proteins might be synthesized, it seems likely that the protein was already preformed in the cell and needed only the addition of pantothenic acid to complete the enzyme system. This would explain the absence of acceleration of fermentation upon the

<sup>7</sup> Unpublished work, Cheldelin *et al.*, Department of Chemistry, The University of Texas.

addition of pantothenic acid to the treated yeast juices. The Fleischmann's yeast is grown in a medium relatively rich in pantothenic acid. In this instance any of the enzyme protein formed would probably be combined with pantothenic acid and none of the pantothenic acid-free protein would be present in the yeast juice. If this were the case, then addition of extra pantothenic acid would be expected to have no effect.

Snell had prepared the sulfonic acid analogue of pantothenic acid, *N*-( $\alpha$ , $\gamma$ -dihydroxy- $\beta$ , $\beta$ -dimethylbutyryl) taurine.<sup>8,9</sup> He has shown that this substance acts antagonistically to pantothenic acid in the latter's effect on the growth of yeast and other microorganisms. Several experiments were carried out to determine if it also acted antagonistically to the effect of pantothenic acid on the fermentation of deficient yeast. No inhibition was observed. On the contrary, when large amounts of the pantoil taurine were added, fermentation was accelerated. The addition of large quantities of the pantoic lactone also had an accelerating effect. It seems possible, therefore, that this unexpected effect of the pantoil taurine was at least partially due to the pantoil radical.

TABLE IV  
*Pantothenic Acid Content of Chick Tissues*

Tissue	Deficient	Normal
Brain .....	23.2 $\gamma$	46.2 $\gamma$
Breast muscle.....	3.2 $\gamma$	12.1 $\gamma$

#### IV

##### *Glycolysis by Chick Tissues Deficient in Pantothenic Acid*

Day old chicks were fed a diet consisting of 74.5 per cent yellow corn meal, 18 per cent washed "vitamin-free" casein, and 7.5 per cent salt mixture supplemented by vitamins as follows: liver extract treated with 10 per cent HCl in methanol equivalent to 20 per cent fresh liver in the diet, 0.2 mg. of thiamin per 100 gm. of diet, petroleum ether extract of 1 gm. of alfalfa leaf meal per 100 gm. of diet, and 2 per cent U.S.P. cod liver oil. This feed had a pantothenic acid content of not more than 0.4 mg. per 100 gm. The experiments were carried out on the tissues when the chicks were 3 to 4 months old. The pantothenic acid analyses on the tissues of the deficient chicks and of chicks fed a commercial diet are given in Table IV. As can be seen, the deficient tissues contained considerably lower content of pantothenic acid than the normal tissues. For all the work reported here the tissues were homogenized according to the method of Potter and Elvehjem.<sup>10</sup>

<sup>8</sup> Snell, E. E., 1941, *J. Biol. Chem.*, **139**, 975.

<sup>9</sup> Snell, E. E., 1941, *J. Biol. Chem.*, **141**, 121.

<sup>10</sup> Potter, V. R., and Elvehjem, C. A., 1936, *J. Biol. Chem.*, **114**, 495.



The effect of pantothenic acid on the oxygen absorption of homogenized breast muscle during the utilization of glucose was determined. A control containing no glucose gave considerably less oxygen absorption, showing that glucose was being metabolized. It can be seen from Table V that pantothenic acid had no effect.

Each flask contained 325 mg. (moist weight) of homogenized muscle in 1.5 cc. of phosphate Ringer solution without sodium carbonate or calcium ion, 0.3 cc. of 2.5

TABLE V  
*Oxygen Consumption of Homogenized Breast Muscle*

Calcium pantothenate	Oxygen per mg. of tissue (dry weight)						
	5 min.	10 min.	20 min.	30 min.	45 min.	105 min.	120 min.
	mm. <sup>3</sup>	mm. <sup>3</sup>	mm. <sup>3</sup>	mm. <sup>3</sup>	mm. <sup>3</sup>	mm. <sup>3</sup>	mm. <sup>3</sup>
0*	0.06	0.11	0.18	0.26	0.36	0.73	0.81
0	0.11	0.17	0.25	0.34	0.45	0.93	1.03
0	0.12	0.18	0.24	0.36	0.44	0.88	0.96
10	0.10	0.15	0.22	0.33	0.44	0.91	0.99
10	0.15	0.20	0.25	0.34	0.43	0.85	0.94

\* Control, contained no glucose.

TABLE VI  
*Anaerobic Glycolysis of Homogenized Brain*

	CO <sub>2</sub> per mg. of tissue (dry weight)			
	15 min.	45 min.	60 min.	Next 15 min.
	mm. <sup>3</sup>	mm. <sup>3</sup>	mm. <sup>3</sup>	mm. <sup>3</sup>
No pantothenic acid.....	0.67	2.15	2.56	0.45
10γ calcium pantothenate .....	0.82	2.13	2.50	0.44
1000γ sodium salt of pantoyl taurine.....	0.75	2.25	2.25	0.58

per cent glucose, 0.1 cc. containing the indicated amount of calcium pantothenate, and 0.15 cc. of 20 per cent KOH in the center cup. An atmosphere of air was used.

The effect of pantoyl taurine as well as that of pantothenic acid itself upon the anaerobic glycolysis of pantothenic acid deficient homogenized chick brain was determined. The results shown in Table VI indicate that neither pantothenic acid nor its sulfonic acid homologue has any effect.

The gray matter of the brain from a deficient chick was homogenized in phosphate Ringer solution and 2.9 cc. containing 200 mg. (moist weight) were pipetted into each flask. To this was added 0.1 cc. of water containing the test substance. The flasks were then placed in the bath and aerated for 5 minutes with a mixture of 95 per cent

nitrogen and 5 per cent carbon dioxide. The mixture was passed over heated copper to remove all traces of oxygen. The sodium D(+) salt of pantoyl taurine was used. Results are shown in Table VI.

A number of other experiments using deficient chick tissues were performed, but in no case could any effect of pantothenic acid, either on the oxygen consumption or the anaerobic glycolysis, be demonstrated. It therefore seems unlikely that pantothenic acid serves as a *dissociable* coenzyme for the glycolytic system.

#### SUMMARY

1. Added pantothenic acid was found to have no appreciable effect on the fermentation of glucose when used in conjunction with preparations of dialyzed yeast maceration juice or acetone-precipitated yeast maceration juice.

2. Addition of pantothenic acid failed to affect the rate of phosphorylation of glucose or the rate of decarboxylation of pyruvic acid by yeast maceration juice.

3. Pantothenic acid showed no effect on the rate of glycolysis by homogenized deficient chick tissues.

4. The accelerating effect of pantothenic acid on fermentation by deficient yeast cells was found to be accompanied by a "binding" of pantothenic acid by the yeast cells.



## THE VALENCE OF CORPUSCULAR PROTEINS\*

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The quantity most directly related to the net charge or valence of corpuscular proteins is the electrophoretic mobility. In 1932 Abramson (1) showed that in solutions of the same ionic strength the electric mobility of the same protein at different hydrogen ion activities is directly proportional to the number of hydrogen ions bound or released as determined by titration curves. His comparisons were made with gelatin, serum albumin, egg albumin, and casein. The generality of Abramson's rule has been confirmed by Daniel (2), Moyer and Abels (3), Moyer and Abramson (4), Longworth (5), and also by Cannan, Palmer, and Kibrick (6). Longworth's data on egg albumin are especially interesting, for his comparison extends from pH 3 to 12. Inasmuch as the results of Abramson and of Moyer seem to have been misunderstood, it should be emphasized that they deal with comparisons of data on *dissolved* proteins. If the protein in the adsorbed state happens to agree, it is incidental to the argument.

Not only did Abramson show proportionality between the electric mobility and titration curves, but he also attempted to calculate the valence of proteins from the available electrophoretic and titration data. The agreement, however, was incomplete.

Although acid-base titration and membrane potential measurements on protein solutions give results that are related to the electric mobility, the actual determination of the net charge from these measurements is subject to difficulties in interpretation and questionable assumptions. On the other hand, an exact interpretation of electric mobility data in terms of net charge requires independent information concerning the size and shape of the protein molecule and the sizes of the other ions in the solution. Information regarding size and shape is not yet available for any protein. Extreme limits of variation of these quantities, however, may be obtained from sedimentation and diffusion data by the assumption, on the one hand, of spherical shape and enough hydration, or, on the other, of enough asymmetry, to account for the diffusion constant.

The results presented in this communication indicate that when these factors are taken into account, values for the valence calculated from titration, electro-

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phoresis, and membrane potential measurements agree closely. It will be shown that for the cases to be considered the uncertainty about size and shape causes a maximum spread of about 20 per cent in the valence determined from mobility data by using the two extreme models: a hydrated sphere and an unhydrated, elongated rod.

### *Theoretical*

In obtaining the valence of proteins from their electrophoretic mobility and size and shape, dependence is placed upon the Debye-Hückel theory (7), modified to include the effect of the ions in the ion atmosphere, and on Stokes' law, as applied to such systems by Henry (8). Under conditions ordinarily encountered, both the extended Debye-Hückel theory and the hydrodynamic assumptions of Stokes' law should apply quite accurately to systems of the corpuscular proteins. Conditions under which considerable deviations from the simplified Debye-Hückel differential equation should occur in protein solutions, due to the Gronwall-La Mer effect (9), have been investigated by Gorin (10). It was concluded as a result of this work that, in the case of proteins of the size of egg albumin or larger and for ionic strengths greater than 0.01, the Gronwall-La Mer correction is small unless the protein is highly charged (valence greater than 10). At ionic strengths greater than 0.1, the correction is negligible for all values of the net charge likely to be encountered in protein systems. For the systems to be presented here, the Gronwall-La Mer effect may be neglected.<sup>1</sup>

The theory to be outlined below applies equally well to protein systems made up of molecules that all have the same charge at any instant or to systems in which the charge of the molecules fluctuates rapidly with time. In the latter case, the net charge determined by electrophoresis is the charge taken over a time average. The one debatable assumption involved in the application of the Debye-Hückel theory to protein systems is that the net charge over a time average behaves as if symmetrically distributed within the molecule or over its surface. However, from the point of view of physical realities there can be little doubt that this is an assumption that is generally justified.

After the net charge of the protein is obtained, within the limits indicated above, it is then possible to compare these values with those obtained from the interpretation of titration data and of membrane potential measurements. As has been the experience of Abramson (1, 12), Moyer and Abels (3), Longsworth (5), and Cannan, Palmer, and Kibrick (6), reached on the basis of more approximate solutions of the Debye-Hückel differential equation, we too find that

<sup>1</sup> Recently, Tiselius and Svensson (11) have claimed that the simple Debye-Hückel theory can be successfully used over a wide range of ionic strengths to calculate the electrophoretic mobility from the net charge estimated by measurements of membrane potentials. See, however, Gorin (10) and Longsworth (5).

values of the net charge calculated from electrophoretic and titration data, at the same ionic strength, agree poorly. For this reason, we have concluded that the ordinary interpretation of titration data is open to serious question and should be reexamined. Steinhardt (13), working on an entirely different basis, has come to similar conclusions.

In the ideal case, assuming that only protons are bound to protein, the valence,  $\nu$ , is equal to the number of protons bound (or released) per molecule of protein. When the valence is multiplied by the charge on an electron ( $e = 4.80 \times 10^{-10}$  E.S.U.), the net charge,  $Q$ , of the protein molecule, over a time average, is obtained. In other words,

$$Q = \nu e = hMe, \quad (1)$$

where  $h$  is the number of equivalents of hydrogen bound per gram of protein of molecular weight,  $M$ . Equation (1) no longer applies when significant numbers of ions other than hydrogen are bound by the protein and, especially, when this binding is dependent on pH.

*The Charged Sphere.*—According to the theories of Gouy and others (14), a charged particle in an ionic medium accumulates in its neighborhood a cloud of ions whose net charge, over a time average, is opposite in sign and equal in magnitude to that existing on the particle. The statistical mean distance of the ions from the surface is referred to as the "thickness of the electric double layer" and will depend on the concentration of ions present. These ions are free to move. Close to the surface, however, there can be a film of tightly held water into which they can rarely pass. Over a time average, the statistical thickness of this film of water will determine the position of the surface of shear. Water molecules inside this boundary are carried with the particle to form a single kinetic diffusing unit. The surface of shear is the "effective" surface of the particle as far as electrophoretic and diffusion effects are concerned. Electric charges inside the surface of shear contribute to the charge on the particle, while those outside constitute the ion atmosphere of the particle.

This non-uniform distribution of charges sets up a potential,  $\psi$ , extending outward to infinity from the surface of shear. At the surface of shear,  $\psi$  assumes the special value,  $\zeta$ . If a particle of radius,  $r$ , is subjected to an applied electric field, it will move with a mobility,  $v$ , that is directly proportional to the  $\zeta$ -potential at its surface. By the theories of Helmholtz, Smoluchowski, and Henry (8), for a particle large enough to obey Stokes' Law,

$$v = \frac{Df(\kappa r)}{6\pi\eta} \zeta, \quad (2)$$

where  $\eta$  is the viscosity of the medium,  $D$  is the dielectric constant, and  $\kappa$  is the well known square root function of ionic strength of Debye and Hückel (7). Values of the Henry factor,  $6/f(\kappa r)$ , are listed in Table I (for the correct ex-

panded form of  $f(\kappa r)$ , see Gorin (10)). Consequently,  $\psi$  can be calculated if  $\zeta$  can be related to the net charge and to the thickness of the electric double layer,  $1/\kappa$ .

Debye and Hückel, following Gouy, combined the Boltzmann and Poisson equations and, with certain simplifying assumptions, arrived at the expression,

$$\nabla^2 \psi - \kappa^2 \psi = 0, \quad (3)$$

from which, on integration, they obtained for a sphere,

$$\psi_i = \frac{Q}{Dr(1 + \kappa d)}. \quad (4)$$

The essential assumptions have been listed elsewhere (15). Here  $Q$  is the net charge of the particle,  $D$ , the dielectric constant of the medium,  $r$ , the radius of

TABLE I  
*The Henry Factor*

$\kappa r$	$6/f(\kappa r)$	$\kappa r$	$6/f(\kappa r)$
0	6.00	5	5.17
1	5.84	10	4.84
2	5.63	25	4.38
3	5.45	100	4.11
4	5.30	$\infty$	4.00

the central particle, and  $d$ , a radial distance called the "distance of closest approach." At this distance,  $\psi$  assumes a special value that we shall designate  $\psi_i$ , rather than  $\zeta$ , its value at the surface of shear. The situation has been diagrammed in Fig. 1. It is  $\zeta$ , rather than  $\psi_i$ , that is needed here. Only when the mean radius of the counter ions in the double layer is zero will  $\psi_i = \zeta$ . At ionic strengths commonly used in protein electrophoresis,  $\psi_i$  will deviate significantly from  $\zeta$ . A correction to equation (4) to include the effect of the size of the ions in the ion atmosphere is therefore necessary.

Values of the radii of the electrolyte ions are available from the equilibrium theory of ionic conductance (16). These values and the equations to be presented below involve the assumption that the charge on the ion is concentrated at its center. This assumption should be valid for simple ions, such as halides and those of the alkali metals. For more complex ions, the assumption may be open to question. In general, electrophoretic results obtained with systems in which simple salts like NaCl make the major contribution to the ionic strength are most suitable for interpretation in terms of net charge on the protein. The systems used by Longworth (5) are especially suitable for this use.

If these notions are introduced into the treatment of Debye and Hückel they lead to the result (15, 16),

$$\zeta = \frac{Q(1 + \kappa r_i)}{Dr[1 + \kappa(r + r_i)]}, \quad (5)$$

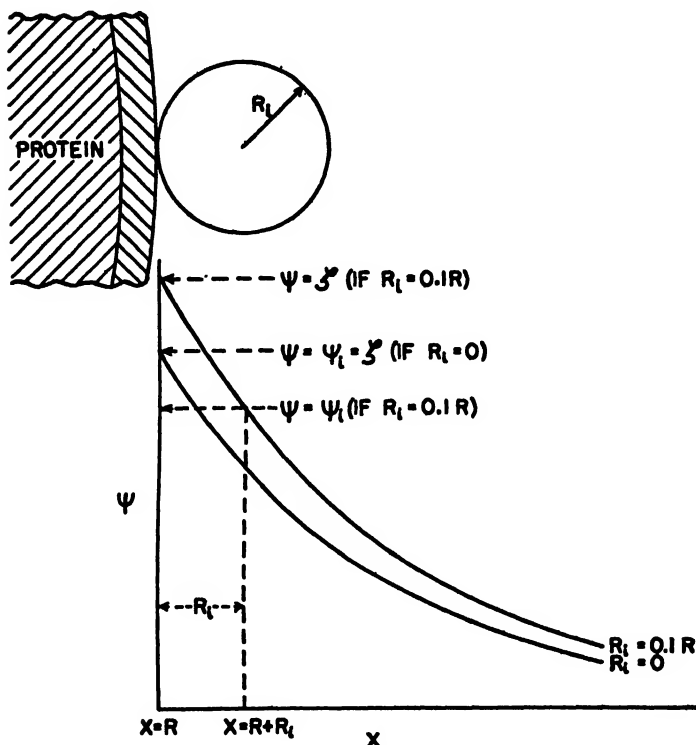


FIG. 1. Diagram of a portion of the surface of a hydrated, spherical protein of radius,  $r$ . An ion of radius,  $r_i$ , is shown at the distance of closest approach. The upper curve represents the fall of potential,  $\psi$ , from the surface of shear as a function of radial distance,  $x$ , calculated by equation (5) on the assumption that  $r_i = 0.1 r$ . Notice that at the surface of shear  $\psi = \zeta$  but that at the distance of closest approach ( $x = r + r_i$ )  $\psi = \psi_i$ . It is  $\zeta$ , rather than  $\psi_i$ , that is directly related to the electric mobility. The lower curve, calculated by equation (4) on the assumption that  $r_i = 0$ , indicates that only in this case does  $\psi = \psi_i = \zeta$  at the surface of shear.

where  $r_i$  is the mean radius of the ions in the diffuse double layer and  $r$  is the radius of the central particle. If the particle is assumed to be hydrated, the effective thickness of its hydration layer (to the surface of shear) must be included in  $r$ . This expression departs most widely from equation (4) at high ionic strengths ( $\mu$ ).



On combining equations (2) and (5), we have, after dividing by the electronic charge,

$$\nu = \frac{6\pi\eta r[1 + \kappa(r + r_i)]}{e \cdot f(\kappa r) \cdot (1 + \kappa r_i)} v. \quad (6)$$

This spherical model predicts the valence from observed values of the mobility under chosen ionic conditions.

*The Unhydrated Charged Cylinder.*—Few, if any, proteins are spherical. Consequently, it seemed more realistic to develop the equations for an elongated cylindrical model (17). In this case, the potential,  $\psi$ , depends only upon the radial distance,  $x$ , from the axis of the cylinder. Equation (3) then becomes

$$\frac{d^2\psi}{dx^2} + \frac{1}{x} \frac{d\psi}{dx} - \kappa^2\psi = 0, \quad (7)$$

a modified Bessel's equation of zero order. When this is solved by the introduction of suitable boundary conditions and by assuming a uniform distribution of surface charge, there is obtained

$$\zeta = \frac{2Q}{D(l + 2a)} \frac{K_0(\kappa a)}{K_1(\kappa a) \cdot (\kappa a)}, \quad (8)$$

where  $e$  is the length and  $a$  the radius of the cylinder.  $K_0$  and  $K_1$  are special Bessel functions of zero and first order respectively (18). The term,  $2a$ , in the denominator is in the nature of an end correction for small values of  $l/a$ . It was chosen to cause equation (8) to join smoothly with equation (11) as  $\mu \rightarrow 0$ .

When the effect of the finite size of the ions in the ion atmosphere is included, the complete equation for  $\zeta$  becomes,

$$\zeta = \frac{2Q}{D(l + 2a)} \left[ \frac{K_0(\kappa a + \kappa r_i)}{(\kappa a + \kappa r_i) K_1(\kappa a + \kappa r_i)} + \ln \left( \frac{a + r_i}{a} \right) \right]. \quad (9)$$

To relate  $\zeta$  to  $v$ , the factor of proportionality,  $C$ , in the general electrophoretic equation,

$$v = \frac{D\zeta}{C\eta}, \quad (10)$$

must be solved for all values of  $\kappa a$ . In the case of a suspension containing elongated protein molecules oriented at random by Brownian movement, we assume that over a time average, one-third lie parallel to each cartesian co-ordinate.<sup>2</sup> Random orientation is justified by the fact that orienting forces involved in electrophoresis are very small when compared with Brownian forces.

<sup>2</sup> This method of averaging is essentially parallel to those used in solving similar physical problems (19, 20).

This method of averaging is simplified by taking one of the coordinates ( $x$ -axis) in the direction of the applied field, whereupon only two cases need be solved. These are: the cylinder migrating parallel to the field and the cylinder migrating perpendicular to the field. For each cylinder parallel to the field, two will be perpendicular. For the parallel case, Henry (8) finds  $C = 4\pi$  for all values of  $\kappa a$ . For the second case,  $C = \pi \cdot F(\kappa a)$ . By an extension of the work of Henry, this function has been obtained by one of us (M. H. G.) by graphical integration (15).<sup>3</sup> The results are given in Table II.

The function,  $F'$ , for a randomly oriented cylinder, corresponding to  $F$  is

$$F' = \frac{2F + 4}{3}.$$

This is also given in Table II.

TABLE II  
*The Constants of Proportionality for a Long Cylinder*

$\kappa a$	Orientation	
	Perpendicular	Random
	$F$	$F'$
0	8.00	6.67
0.4	7.73	6.49
1.0	7.36	6.26
1.4	6.87	5.91
2.0	6.51	5.67
2.4	6.34	5.56
3.0	6.10	5.40
$\infty$	4.00	4.00

It is somewhat more convenient to define the cylinder in terms of its asymmetry and volume rather than with respect to its length and radius. We define asymmetry as  $S = l/2a$ , so that

$$a = \sqrt[3]{v/2\pi S},$$

where  $v$  is the molecular volume.

The electric mobilities of cylinders of varying asymmetries are compared in Table III with the electric mobilities of a sphere of the same molecular volume and net charge. At  $\mu = 0$ , the values of the ratios of the mobilities are exactly equal to the corresponding ratios of the diffusion constants,  $D/D_0$ , of the cylinder

<sup>3</sup> The function was inadvertently attributed in this paper to a parallel cylinder rather than to a perpendicular one.

and sphere respectively. Since the electrophoretic mobilities for cylinders cannot properly be extrapolated to  $\kappa = 0$ , the values in the table at  $\mu = 0$  are calculated for prolate ellipsoids from Perrin's equation (21),

$$\frac{f_0}{f} = \frac{D}{D_0} = \frac{S^{3/2} \ln \frac{1 + \sqrt{1 - S^2}}{S}}{\sqrt{1 - S^2}}, \quad (11)$$

where  $S$  is the ratio of the long to the short axis and  $f/f_0$  is the force factor of the molecule. Notice in the table that the values of the ratios computed by the two methods join smoothly. The values are nearly unaffected by changes in molecular weight between 30,000–150,000. Asymmetry, however, exerts a noticeable effect on the retardation due to the ion atmosphere. It should be emphasized that these calculations are for randomly oriented cylinders of constant net charge but not of constant charge density.

TABLE III  
*Electrophoretic Mobilities of Cylinders*

Asymmetry $l/2a$	Ratio of mobility to that of a sphere of the same molecular volume				
	$\mu = 0$	$\mu = 0.005$	$\mu = 0.02$	$\mu = 0.1$	$\mu = 0.2$
2.5	0.920	0.856	0.774	0.738	0.715
5.0	0.800	0.715	0.653	0.624	0.604
10.0	0.648	0.545	0.503	0.490	0.485
22.5	0.477	0.379	0.361	0.362	0.357
45.0	0.350	0.263	0.254	0.256	0.259

### Methods

Serum albumin B was crystallized from horse serum by the methods of Kekwick (22). The dialyzed product was titrated in a nitrogen atmosphere by the use of a micro burette and a glass electrode. The standards of pH employed were those recommended by MacInnes, Belcher, and Shedlovsky (23). To eliminate the effect of protein concentration as much as possible, the measurements were performed at about 0.25 per cent protein. In those titrations that were performed at constant ionic strength, the proper amount of NaCl was added in advance to the HCl and NaOH used in titrating as well as to the protein solution. The mean ionic activity coefficient was taken as that prevailing in the absence of protein at the ionic strength under consideration. Suitable methods for the calculation of the equivalents of hydrogen bound or released have been discussed by Kekwick and Cannan (24) and by Moyer and Abels (3).

### RESULTS

*Egg Albumin.*—Longworth (5) has found that the diffusion constant,  $D$ , of egg albumin is  $3.99 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$  at  $0^\circ \text{C}$ . If the molecule is assumed to be a

hydrated sphere, its effective radius can be calculated from the Stokes-Einstein equation,

$$\frac{kT}{6\pi\eta D} = 27.8 \text{ \AA.}$$

Longworth has also presented moving boundary data for dissolved egg albumin at 0°C. and  $\mu = 0.1$ . Under these conditions,  $\kappa r = 2.87$ . From Table I,  $6/f(\kappa r)$  is found to be 5.47. On taking the average radius of the electrolyte ions,  $r_i$ , as 2.5 Å.,  $\kappa r_i$  becomes 0.258. If these values are substituted in equation (6), we obtain the valence,  $\nu$ , in terms of  $v$  (in  $\mu/\text{sec.}/\text{volt}/\text{cm.}$ ),

$$\nu = 17.5 v. \quad (12)$$

If, however, the other extreme, an unhydrated cylinder, is assumed, the asymmetry is determined (25) from the diffusion constant of the protein by the use of equation (11). If the molecular weight of egg albumin is 45,000 (26), its unhydrated molecular volume corresponds to that of a sphere of 23.6 Å. The force factor,  $f/f_0$ , is thus  $27.8/23.6 = 1.18$  and the asymmetry is 4/1 for this rod-like model. We first calculate the valence of the unhydrated sphere for  $\kappa r = 2.44$  and  $6/f(\kappa r) = 5.55$ . This gives  $\nu = 13.65 v$ . Reference to Table III shows that a cylinder of this degree of asymmetry should have a mobility equal to 65 per cent of that of a sphere of the same molecular volume. Hence

$$\nu = \frac{13.65}{0.65} v = 21.0 v. \quad (13)$$

Comparison of equations (12) and (13) shows that a difference of about 20 per cent should be found between a sphere and a cylinder of the same diffusion constant under these conditions. Accurate determinations of the valence from titration data would make possible a decision between the effects of water of hydration and asymmetry.

In Fig. 2 are shown the results discussed above, together with curves for the valence calculated by equation (1) from the recent titration data of Cannan, Kibrick, and Palmer (27) at infinite dilution of egg albumin. The titration data have been shifted vertically so that the curves coincide with the mobility curve at the isoelectric point. The low temperature coefficient of the dissociation of carboxyl groups makes possible a comparison of these curves without temperature correction in the range of pH shown in the figure (5, 27). Although quantitative agreement exists between the mobility data and the titration curve in the absence of added salt, it is evident that the values of the net charge calculated from the titration data at the ionic strength of the electrophoretic measurements are several hundred per cent higher.

Tiselius and Svensson (11) have determined the electrophoretic mobility of

egg albumin in phosphate buffers at  $0^{\circ}\text{C}$ . and  $\mu = 0.1$ . Under these conditions, the protein is highly negative and  $r_i$  can still be taken as  $2.5 \text{ \AA}$ ., in spite of the presence of phosphate ions of relatively larger radii. Applying equations (12) and (13) we obtain  $\nu = -11.9$  and  $\nu = -14.1$  for sphere and cylinder respectively.

Adair and Adair (28) obtained  $\nu = -13.8$  for the valence of egg albumin at  $0^{\circ}\text{C}$ . in phosphate buffers at pH 7.1 by means of membrane potential measure-

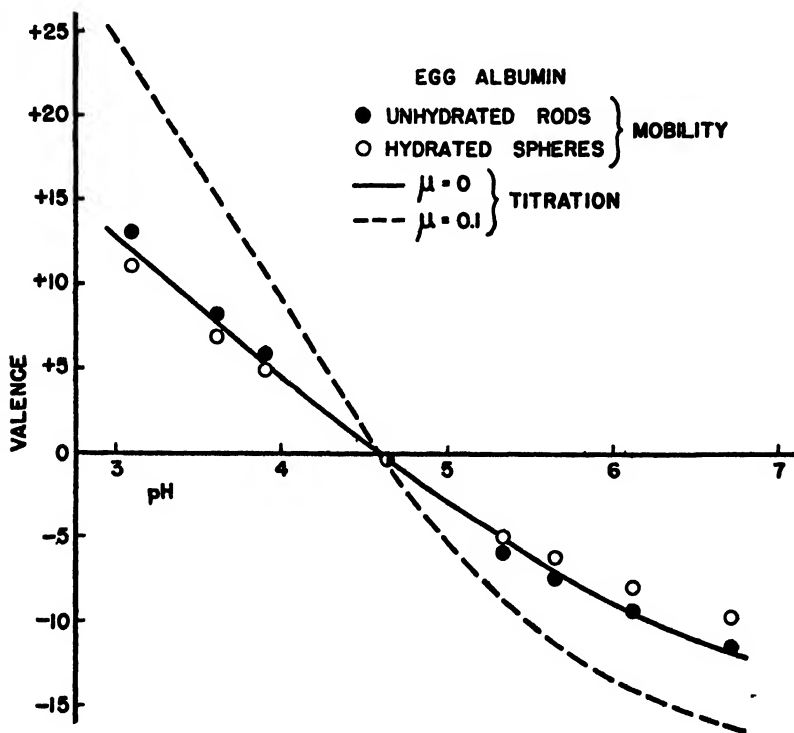


FIG. 2. The valence of egg albumin from titration data of Cannan, Kibrick, and Palmer and electrophoretic results of Longworth. The curves have been shifted to the isoelectric point at pH 4.60.

ments. Thus the membrane potential measurements of Adair and Adair and the extrapolated titration curve lead to similar results, namely that the agreement is somewhat better for the cylindrical than for the spherical model. By inference, it may be concluded that our method to obtain the net charge from extrapolated titration curves and the membrane potential method should lead to very nearly the same results for the same system. No data are available, as yet, to permit a direct comparison of the two methods with the same system.

The titration method is unreliable for points far removed from the isoelectric point. In addition, the isoelectric point of egg albumin is not accurately known in phosphate buffers.

Calculations from the earlier results of Tiselius (29) for egg albumin in acetate

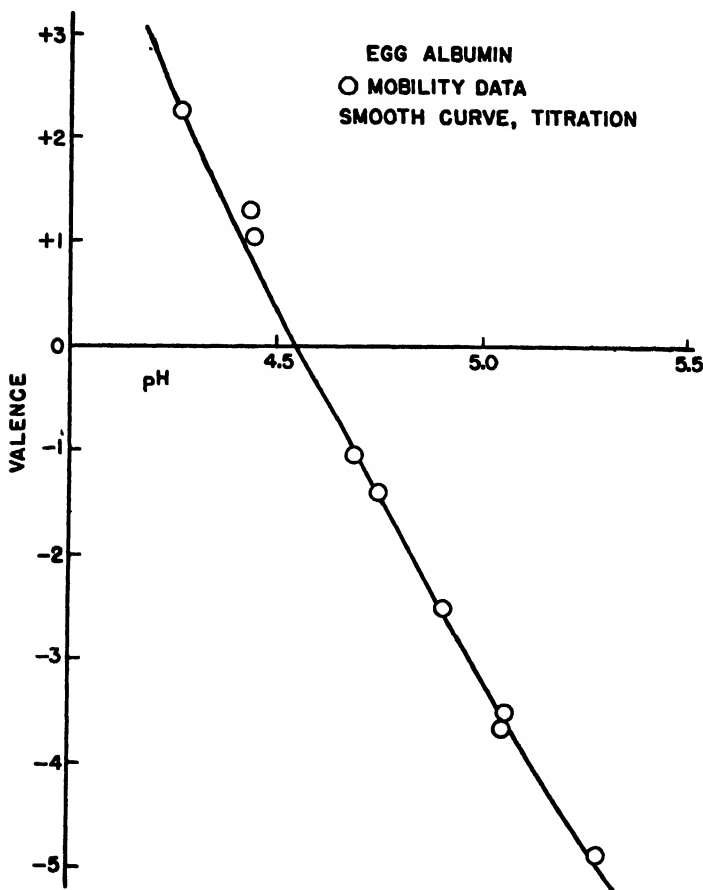


FIG. 3. The valence of egg albumin from titration data of Cannan, Kibrick, and Palmer and electrophoretic results of Tiselius. The isoelectric point lies at pH 4.55 at this ionic strength (0.02). Results for cylinder and sphere have been averaged, as discussed in the text.

buffers at  $\mu = 0.02$  and  $20^\circ\text{C}$ . are shown in Fig. 3. Under these conditions, we obtain  $\nu = 6.9$  and  $\nu = 8.0$  for sphere and cylinder, respectively. Since the electrophoretic measurements probably have an error of the order of 10 per cent, no attempt was made to distinguish between the two models in this case. The plotted points are the average values of the charge calculated for a sphere and

cylinder, namely,  $\nu = 7.45 v$ . The smooth curve is the valence calculated from the titration curve (27) at infinite dilution, shifted vertically to  $v = 0$  at pH 4.545. Notice the excellent agreement between valence from electrophoresis and valence from titration.

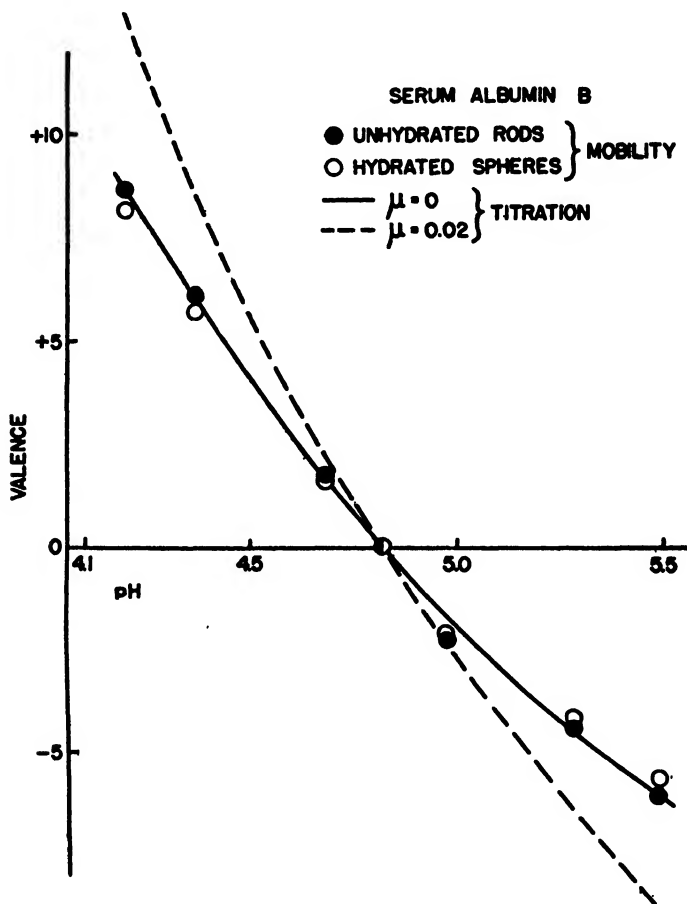


FIG. 4. The valence of serum albumin B from titration data (this paper) and electrophoretic results of Kekwick. The curves have been shifted to the isoelectric point at pH 4.81 ( $\mu = 0.02$ ).

*Serum Albumin.*—In Fig. 4 are shown values for the valence of serum albumin B calculated from the electric mobility data of Kekwick (22) at 20°C. and  $\mu = 0.02$ . He finds 70,000 for its molecular weight and  $6.10 \times 10^{-7} \text{cm.}^2 \text{sec.}^{-1}$  for its diffusion constant at 20°C. These data lead to an asymmetry of 5/1, if an unhydrated rod is assumed. The titration curves are our own results. Here,  $\nu/v = 9.65$  for a sphere or 10.31 for a rod.

Fig. 5 shows a similar comparison for this protein at  $\mu = 0.1$ . In this instance, the mobility data of Moyer and Moyer (30), for films of serum albumin B adsorbed on microscopic particles, were used. Inasmuch as the electric mobility data at an ionic strength of 0.02 for this protein adsorbed on quartz or

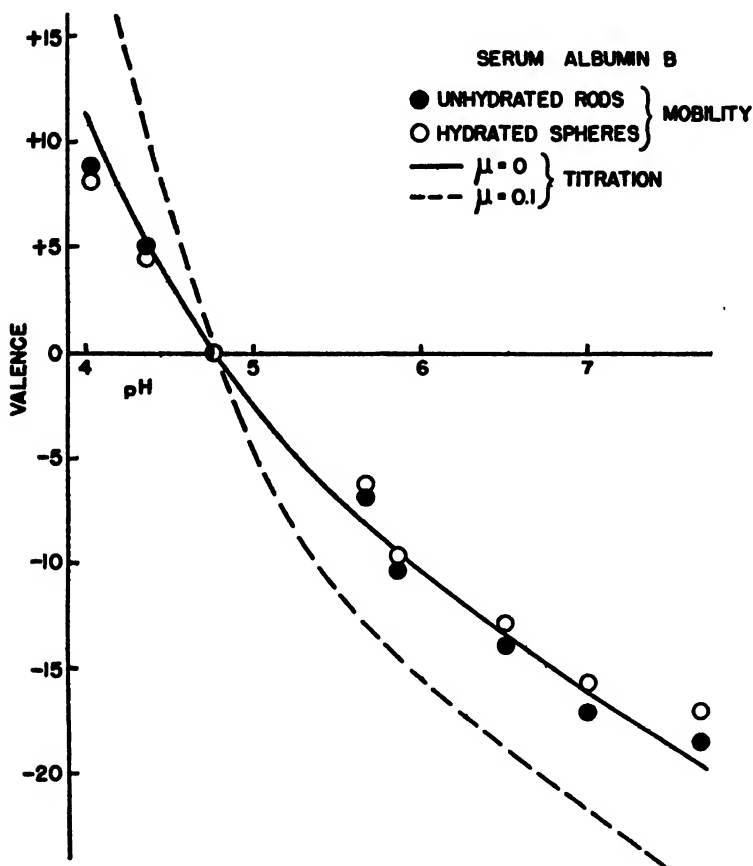


FIG. 5. The valence of serum albumin B from titration data (this paper) and electrophoretic results of Moyer and Moyer. The curves have been shifted to the isoelectric point at pH 4.76 ( $\mu = 0.1$ ).

collodion particles have been shown (30, 31) to be in agreement with moving boundary measurements on dissolved serum albumin B, it is highly probable that the data in Fig. 5 correspond to the behavior of the dissolved protein at  $\mu = 0.1$ . The titration curves in Figs. 4 and 5 follow a course with respect to the mobility data similar to that found for egg albumin. In this case,  $\nu = 12.75 \nu$  for a sphere or  $13.94 \nu$  for a rod.



***$\beta$ -Lactoglobulin.***—Similar calculations have been made for  $\beta$ -lactoglobulin, by using the electric mobility data of Pedersen (32) at 20°C and  $\mu = 0.02$  and the titration data of Cannan, Palmer, and Kibrick (6). Oncley (33) suggests 40,000 as the most probable value for the molecular weight. This figure, combined with the diffusion constant,  $7.27 \times 10^{-7} \text{cm}^2 \text{sec}^{-1}$ , found by Polson (34) at 20°C., yields a force factor of 1.26, which corresponds to an asymmetry of

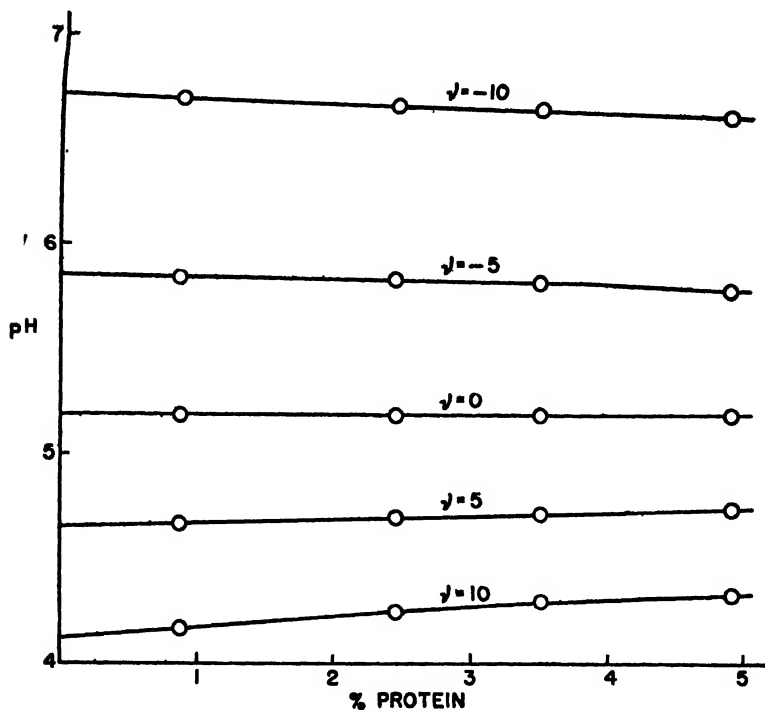


FIG. 6. Extrapolation of the titration data of Cannan, Palmer, and Kibrick on  $\beta$ -lactoglobulin in the absence of salt to infinite dilution of protein.

5.2, if an unhydrated cylinder is assumed. This yields  $\nu = 8.11v$ . If, however, a hydrated sphere is chosen as the model,  $\nu = 7.35 v$ .

Cannan, Palmer, and Kibrick present two sets of titration data for this protein: (1) in the presence of various concentrations of salt at a protein concentration of 0.5 per cent and (2) at various concentrations of protein in the absence of added salt. A curve representing the titration curve at infinite dilution of electrolytes and protein has been obtained by extrapolation of this latter set of results (Fig. 6). At values of pH below 4, the extrapolation becomes less certain; in addition, the steadily increasing concentration of  $\text{Cl}^-$  begins to con-

tribute to the ionic strength. Hence we have decided to limit our considerations to regions above this value.

Fig. 7 shows our results for this protein. As before, the agreement with the electrophoretic results is good only for the extrapolated titration curve in the absence of salt. Likewise, the correspondence is somewhat better for the cylindrical than for the spherical model.

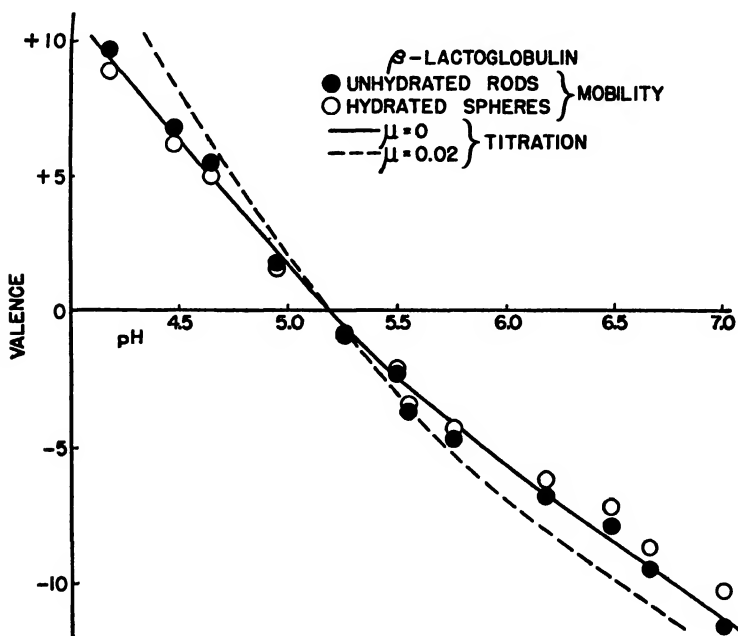


FIG. 7. The valence of  $\beta$ -lactoglobulin from titration data of Cannan, Palmer, and Kibrick and electrophoretic results of Pedersen. Inasmuch as both isoionic and isoelectric points are at pH 5.19, no adjustment of the curves was necessary.

#### DISCUSSION

The chief difficulty encountered in determining the valence of proteins from titration curves involves the interpretation of titration data. It is unlikely, especially at higher values of ionic strength, that the titration curve represents binding or release of hydrogen ions alone. As a first approximation, the procedure of shifting the titration curve vertically to account for the binding of ions other than hydrogen and hydroxyl appears to be justified for pH values not far removed from the isoelectric point. With a change in ionic strength, the influence of other ions, such as  $\text{Na}^+$  or  $\text{Cl}^-$ , upon the net charge of the protein results mainly in a vertical shift of the net charge curve without much change in its slope; *i.e.*, a shift in isoelectric point (3, 4, 12, 15, 35). Results suggest that

over the pH range investigated the binding of ions other than hydrogen to protein is relatively independent of pH. Two types of interactions of these ions with protein would be expected to be nearly independent of pH. One is adsorption of ions like  $\text{Na}^+$  or  $\text{Cl}^-$  by non-polar parts of the molecule. The other is ion pair formation between the electrolyte ions and the charged groups of the protein molecule (36). Ion pair formation should be independent of pH only in the neighborhood of the isoelectric point, where the net charge of the protein is small compared to the total charge. We have therefore restricted our considerations to this region.

Steinhardt (13), moreover, has obtained results which indicate that undissociated acid, such as HCl, is bound to protein along with hydrogen ion. This is equivalent to saying that ion pairs of  $\text{H}^+\cdot\text{Cl}^-$  tend to be taken up by the protein molecule. Under conditions where this effect is appreciable, the titration curve would yield too high a value for the valence, since the binding of molecular HCl would remove hydrogen ions from solution and contribute to the experimental titration curve but should not affect the net charge. Hence it would not affect the electrophoretic mobility.

Let us consider the binding of hydrogen ions involving only the acidic and basic groups of the protein molecule. In this case, if we make the assumption that this binding is essentially independent of ionic strength, it is possible to obtain what may be considered the *intrinsic* net charge curve (the titration curve concerned with the binding of hydrogen ions alone), by extrapolating the experimental titration curve to zero ionic strength and zero protein concentration. By hypothesis, this curve would apply at all values of ionic strength. To correct for the binding of other ions, such as  $\text{Cl}^-$ , the titration curves were shifted vertically, so that the zero point for acid or base bound corresponded with the isoelectric point determined by electrophoresis.

As Abramson (12) has suggested, it seems likely that, as results on additional proteins become available, individuals similar to gelatin, whose isoelectric point is hardly shifted by ionic strength, will be encountered. It is possible that  $\beta$ -lactoglobulin is one of these cases, for its isoionic point is unaffected by salt (Fig. 7) and is identical with its isoelectric point, at least at  $\mu = 0.02$ . A comparison of the valence curves is possible, therefore, under conditions that are free from any question as to the validity of the vertical adjustment.

The experimental titration curves, however, deviate very markedly from the ideal one at higher values of ionic strength and protein concentration. Cannan, Kibrick, and Palmer (6, 27) have attributed these deviations to the effect of salt on the dissociation constants of the amino and carboxyl groups, combined with an activity effect on the composite protein ion. On the other hand, we prefer, as a first approximation, to attribute these effects to the binding of undissociated acid to the proteins. The magnitude of this binding would depend on the activity product of anions and hydrogen ions in the solution and

would therefore be a function of pH, salt concentration, and protein concentration. In basic solutions binding of pairs, such as  $K^+$  and  $OH^-$ , to protein may also occur.

It will be recalled that values of the net charge of protein molecules obtained from electrophoretic data by the use of the two extreme models, the elongated rod and the hydrated sphere, agree within 15 to 20 per cent. Furthermore, in each case they agree very well with the intrinsic net charge curve for values of the valence obtained from titration data by means of our assumptions. On the other hand, the dashed lines, which represent the net charge calculated on the assumption that no molecular HCl is bound to protein, deviate widely from the other results.

The agreement between the rod-like model and the titration data is somewhat better than that for the sphere but it should not be concluded that these proteins are unhydrated rods instead of hydrated spheres. It is probable that the proper model lies somewhere between these extremes; *i.e.*, a less extended rod with some hydration.

#### SUMMARY

By the use of two extreme models: a hydrated sphere and an unhydrated rod the valence (net charge) of corpuscular proteins can be successfully calculated from electric mobility data by the Debye-Hückel theory (modified to include the effect of the ions in the ion atmosphere) in conjunction with the electrophoretic theory of Henry. As pointed out by Abramson, this permits a comparison with values for the valence from titration data.

Electrometric titration measurements of serum albumin B (Kekwick) have been determined at several ionic strengths. These results, together with the available data in the literature for serum albumin B, egg albumin, and  $\beta$ -lactoglobulin have been used to compare values for the valence calculated from measurements of titration, electrophoresis, and membrane potentials. The results indicate that the usual interpretation of titration curves is open to serious question. By extrapolation of the titration data to zero ionic strength and protein concentration, there results an "intrinsic" net charge curve describing the binding of  $H^+$  ( $OH^-$ ) ion alone. This curve agrees closely, in each case, with values of the valence calculated from mobility data (which in turn are in close accord with those estimated from membrane potential measurements). The experimental titration curves in the presence of appreciable quantities of ions and protein deviate widely from the ideal curve. It is suggested that, under these conditions, binding of undissociated acid (base) leads to erroneous values for the net charge. This binding would not affect the electrophoretic mobility.

Values of the net charge obtained by the two extreme models from electrophoretic data are in agreement within 15 to 20 per cent. The agreement be-

tween the cylindrical model and the titration data is somewhat better in each case than with the sphere; *i.e.*, this comparison enables a choice to be made between asymmetry and hydration in the interpretation of results from sedimentation and diffusion measurements on proteins. It is concluded that the proteins discussed here are somewhat asymmetric and also hydrated.

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# THE ACTION OF SULFONAMIDES ON THE RESPIRATION OF BACTERIA AND YEAST

## INHIBITION OF BACTERIAL AND YEAST CARBOXYLASES BY SULFONAMIDE DRUGS STRUCTURALLY RELATED TO COCARBOXYLASE\*

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In the previous three communications on the mechanism of action of sulfanilamide, Sevag and Shelburne (1) reported that this drug primarily blocked bacterial cell respiration in the absence as well as in the presence of active growth. The inhibition of aerobic and anaerobic respiration resulted in the proportional inhibition of growth under the conditions used. *p*-Aminobenzoic acid often reversed partly or completely the inhibition of respiration by sulfanilamide.

On the basis of the above findings and those of others reviewed previously (1), a theory correlating the drug action with the similarity of chemical structures of the drugs and the coenzymes was formulated. It was stated that the chemotherapeutic substances which have structural similarity to the whole, or part of the coenzyme molecules may combine specifically with the protein carrier of the respiratory enzymes—the result being the displacement of the coenzymes by the drug and the formation of an inactive “enzyme-analogue” or the formation of a hypothetical “drug-protein-coenzyme” complex. These being incapable of oxidizing glucose, bacterial respiration and growth are inhibited. In advancing this theory reservations were made to account for the activity of such drugs as do not possess structural similarity with the known coenzymes. In such cases mutual affinity between the drug and the specific enzyme proteins was considered as a possible determining factor.

In accordance with the above theory sulfapyridine, for example, with its pyridine ring was mentioned as a potential competitor of cozymase I and II, and sulfathiazole (likewise sulfadiazine) of cocarboxylase. These coenzymes are present in the respiratory systems of most living cells. They, therefore, offer the first point of attack in determining which link in the chain of reactions associated with the metabolism of glucose is affected most by a given sulfonamide drug.

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\* Article IV in a series on the mechanism of sulfonamide action. The present study was conducted under grants from the Josiah Macy, Jr., Foundation and Merck and Company.



Bacterial and yeast carboxylase systems were chosen for this study as one of the means to test and corroborate the implications of the above theory. The decarboxylation of pyruvic acid by carboxylase is of almost universal occurrence in living cells. This reaction, carried out under anaerobic conditions, is principally a one-step reaction, and is relatively free from the interference of side or parallel reactions. The effect of the drugs will be, therefore, directly on the carboxylase system. To obtain clearer insight into the relative importance of various rings and radicals in the molecules of the drugs in inhibiting the carboxylase system, the widely used sulfonamide drugs, their homologues, and their simple components were studied comparatively.

### *The Enzyme Systems Studied*

**DETERMINATION OF THE CARBOXYLASE ACTIVITY OF BACTERIA AND YEASTS.**—Pneumococcus Type 1, *Streptococcus pyogenes* (C203M), *Staphylococcus aureus*, *Escherichia coli*, and brewer's and baker's yeast were studied to determine the optimal conditions of their carboxylase activity. The experiments were carried out anaerobically in an atmosphere of 95 per cent nitrogen and 5 per cent CO<sub>2</sub> in the presence of sodium pyruvate solution. The CO<sub>2</sub> evolved was measured manometrically with a Barcroft-Warburg set-up. All of the gas evolved was absorbable if KOH was placed in the inset tube of the vessel, showing the evolved gas to be CO<sub>2</sub>.

*Pneumococcus Type 1.*—Suspensions of Pneumococcus Type 1, washed and unwashed, grown for 4 and 16 hours in serum broth, in the presence and absence of catalase (to eliminate H<sub>2</sub>O<sub>2</sub> formed during growth), were tried out in three separate experiments at pH 6. In no case did decarboxylation of pyruvate take place. In parallel experiments the addition of 1, 5, 10, and 100γ of cocarboxylase and of 0.1 mg. of Mg did not produce any activity. Decarboxylation of pyruvic acid by pneumococci (2) evidently takes place by a different route and will be discussed later.

*Streptococcus pyogenes.*—In contrast, similar experiments with *Streptococcus pyogenes* (C203M) grown for 16 hours in serum broth showed a residual carboxylase activity. This activity was markedly augmented by the addition of cocarboxylase; 33 c.mm. of CO<sub>2</sub> were evolved in the absence of cocarboxylase while 124 c.mm. of CO<sub>2</sub> were evolved when 100γ of cocarboxylase were added. In a second experiment a dense suspension of streptococci grown for 16 hours did not show any activity in the presence of 0.0517 M pyruvate, but the addition of 25γ of cocarboxylase caused the evolution of 72 c.mm. of CO<sub>2</sub> within 150 minutes. An increase of 50 to 100γ of cocarboxylase did not improve this activity. The activity being too small, this organism was not further employed in the present study.

*Staphylococcus aureus.*—Fresh suspensions of *Staph. aureus* grown for 4 to 16 hours exercised uniform carboxylase activity, 4 mg. of cocci producing 400 c.mm. of CO<sub>2</sub> in 120 minutes. The dry weight of bacteria was estimated by micro-Kjeldahl nitrogen determination. The nitrogen values were multiplied by 7.2 as the protein factor, assuming that the organism contains 14 per cent nitrogen.

*Carboxylase Activity before and after Drying Staphylococcus aureus from the Frozen State.*—To have uniform carboxylase activity, it is necessary that the weight of the samples used in the daily experiments be identical. Samples of *Staph. aureus* dried

from the frozen state proved satisfactory. These organisms were twice washed with M/15 phosphate buffer of pH 7.16 before drying. The results of a comparative

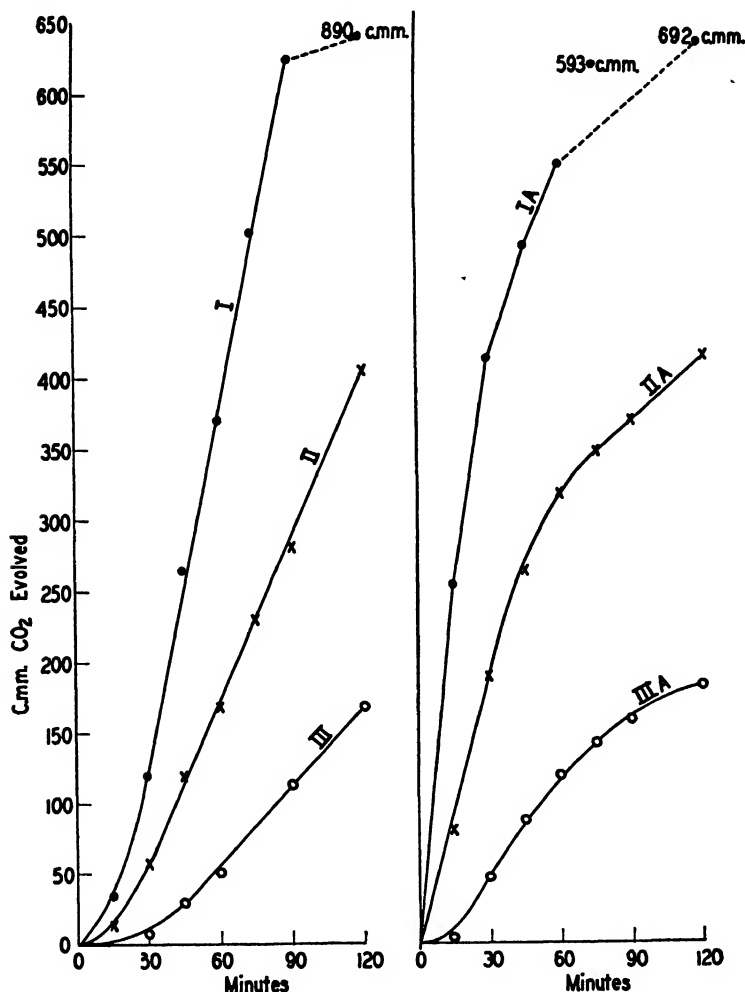


FIG. 1. Decarboxylation of pyruvic acid in *Staph. aureus*. Curves I, II, and III represent the activity of 14.4, 7.2, and 3.6 mg., respectively, of fresh *Staph. aureus*. Curves IA, IIA, and IIIA represent the activity of 14.4, 7.2, and 3.6 mg., respectively, of staphylococci dried from the frozen state.

study of the fresh and the corresponding dried samples of equal weights (Fig. 1) show that on drying from the frozen state staphylococci experience practically no loss of carboxylase activity. The addition of 1 to 10 $\gamma$  of cocarboxylase to the reaction system did not increase the activity of staphylococci.

*Optimal Carboxylase Activity of Dried Staphylococci.*—Experiments were carried

out to determine the optimal activity as a function of pH and as a function of concentration of substrate. The results are given in Table I.

*Removal and Restoration of Carboxylase Activity of Staphylococcus aureus.*—To test whether the CO<sub>2</sub> evolved was due to carboxylase the following experiments were carried out. Fresh suspensions or suspensions of staphylococci dried from the frozen state were treated according to the method of Lohmann and Schuster (3) to remove cocarboxylase. Pure cocarboxylase (Merck) was then added and the de-

TABLE I  
*Carboxylase Activity of Dried Staphylococcus aureus As a Function of pH and of Substrate Concentration*

pH*	CO <sub>2</sub> /2 hrs.	Pyruvate	CO <sub>2</sub> /2 hrs.†
	<i>c.mm.</i>		<i>c.mm.</i>
7.28	284	0.017 M	231
7.10	279	0.034 M	332
6.54	380	0.052 M	399
6.13	416	0.068 M	492
		0.10 M	430

\* The system contained 0.3 cc. (26.4 mg.) pyruvate in a volume of 5.8 cc. (0.0517 M). Weight of organisms per vessel 0.55 mg. of nitrogen, or 3.96 mg. of dry cocci. Phosphate M/125. Temperature 37.5° C.

† The experiments were carried out at pH 7.16 in consideration of the inhibition experiments with drugs which have greater solubility at this pH than at the pH of optimal activity. The reaction system was identical with that used in pH experiments except for varying quantities of pyruvate.

TABLE II  
*Carboxylase Activity of Staphylococcus aureus before and after Treatment*

Untreated	Treated with M/10 Na <sub>2</sub> HPO <sub>4</sub>	Washed once with saline	Loss of activity
<i>c.mm. CO<sub>2</sub></i>	<i>c.mm. CO<sub>2</sub></i>	<i>c.mm. CO<sub>2</sub></i>	<i>per cent</i>
508	245	—	52
721	466	—	36
552	—	297	46

carboxylation of pyruvate was measured. Treating the fresh suspensions of 4 hour staphylococci with M/10 Na<sub>2</sub>HPO<sub>4</sub>, according to Lohmann and Schuster, did not noticeably affect the carboxylase activity. A sample which liberated 862 c.mm. of CO<sub>2</sub> before treatment liberated 798 c.mm. of CO<sub>2</sub> after treatment, (7 per cent reduction of activity). With the addition of 10γ of cocarboxylase to the treated sample, 843 c.mm. of CO<sub>2</sub> were evolved.

On the other hand, if the staphylococci are dried from the frozen state and then washed with either M/10 Na<sub>2</sub>HPO<sub>4</sub> or 0.85 per cent NaCl the activity is reduced about 50 per cent (Table II). The lost activity is nearly completely restored by the addition of cocarboxylase. The activity of M/10 Na<sub>2</sub>HPO<sub>4</sub> treated samples was restored to

76-85 per cent of the original on adding 100 $\gamma$  of cocarboxylase. The activity of the saline washed sample was restored to 92 per cent of the original with 50  $\gamma$  of cocarboxylase.<sup>1</sup>

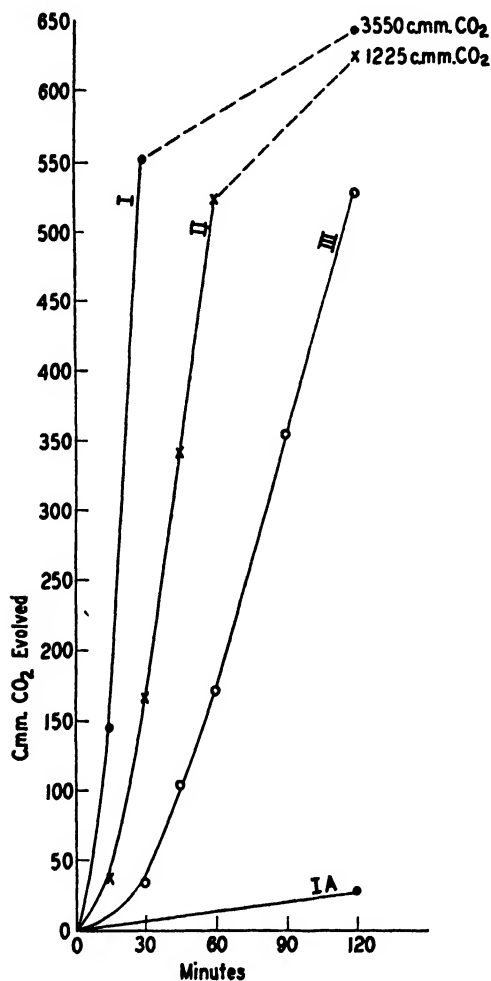


FIG. 2. Decarboxylation of pyruvic acid in *E. coli*. Curves I, II, and III represent the activity of 4.32, 1.73, and 0.86 mg., respectively, of fresh *E. coli*. Curve IA represents the activity of 4.32 mg. of *E. coli* dried from the frozen state.

<sup>1</sup> The aerobic metabolism of pyruvic acid in *Staph. aureus* has previously been investigated (4, 5). In connection with the present study the reader is referred to a paper by Krebs (6) on the anaerobic dismutation of pyruvic acid in *Staph. aureus* and *Staph. albus*. According to Krebs, following the decarboxylation of one mole of pyruvate the resulting acetaldehyde with a second mole of pyruvate metabolizes to

**Carboxylase Activity of *Escherichia coli*.**—In contrast to *Staph. aureus*, the suspensions of *E. coli* grown in glucose broth for 16 hours, twice washed with saline, and dried from the frozen state, were found completely inactive. When washed with M/15 phosphate buffer at pH 6, and then dried from the frozen state, a sample of 2.2 mg. of *E. coli* liberated 234 c.mm. of CO<sub>2</sub> in the presence of 0.0517 M pyruvate, but in later experiments the dried organisms had practically lost their activity. In all the daily inhibition experiments, therefore, fresh suspensions of *E. coli*, twice washed with M/15 phosphate buffer of pH 7.1 and standardized with a photoelectric turbidometer, were used. In an experiment the carboxylase activity of the fresh and dried suspensions of equal *E. coli* content was measured comparatively. The results are given in Fig. 2.

**Yeast and Carboxylase prepared from Yeast.**—The carboxylase activities of air-dried 20 mg. samples of three different yeasts were measured at pH 6.23. The CO<sub>2</sub> evolved during a period of 90 minutes was as follows: brewer's fresh bottom yeast, 752 c.mm.; baker's yeast, 575 c.mm.; ale yeast, 531 c.mm.

Using the method of Green, Herbert, and Subrahmanyam (7) the carboxylase prepared from an old batch of air-dried brewer's yeast had little activity; a preparation from ale yeast was inactive. A preparation from fresh air-dried brewer's yeast had a fair activity. This solution after dialysis contained 0.256 mg. of N/cc. No attempt was made to purify this preparation by fractionation for inhibition experiments. 0.6 cc. of preparation containing 0.15 mg. of N evolved 90 c.mm. of CO<sub>2</sub> at pH 6 within 3 minutes. At pH 7.16, 0.2 cc. of a sample (20 cc. sample dialyzed overnight against 1 liter of distilled water) liberated 161 c.mm. of CO<sub>2</sub> within 60 minutes in comparison to a non-dialyzed sample which liberated 190 c.mm. of CO<sub>2</sub>. Under identical conditions 5 mg. of air-dried fresh brewer's yeast liberated 234 c.mm. of CO<sub>2</sub>.

#### *Inhibition of Carboxylase by Sulfonamide Drugs and Their Derivatives*

The inhibitory effect of sulfonamide drugs and derivatives on the carboxylase activity of *Staph. aureus*, *E. coli*, baker's and brewer's yeast, and a carboxylase

lactate and acetate, and this reaction is the preferential one. Studying this reaction aerobically he found that 0.1 M of pyruvate consumed 328 c.mm. O<sub>2</sub>, and anaerobically evolved 249 c.mm. CO<sub>2</sub> within a 95 minute period. In our experiments 0.1 M of pyruvate consumed 52 c.mm. O<sub>2</sub> and evolved 651 c.mm. total CO<sub>2</sub> (free and bound as NaHCO<sub>3</sub>), within a 120 minute period. Anaerobically 0.1 M pyruvate evolved 430 c.mm. CO<sub>2</sub>. In parallel experiments, the oxygen consumed by 0.1 M pyruvate was 52 c.mm. in contrast to 286 c.mm. O<sub>2</sub> consumed by 0.05 M lactate. These results show that the dismutation of pyruvate to lactate progresses at a very much slower rate than the decarboxylation of pyruvate. Within a 2 hour period, under the conditions of our experiments, not more than 10–20 per cent lactate could have been formed. This small percentage of dismutation of pyruvic acid to lactic acid does not introduce an error into the interpretation of the results of the present study from the point of view of the action of sulfonamides on the activity of carboxylase.

The discrepancy between our results and those of Krebs is of quantitative nature and not one of principle. This is no doubt due to the difference of strains of *Staph. aureus* used, conditions of culture, and of experiments. Such differences in bacterial metabolic activity have been reported frequently (2, 5).

TABLE III

Per Cent Inhibition of the Carboxylase Activity of Bacteria and Yeast by Sulfonamide Drugs

Carboxylase systems	No drug c.mm. CO <sub>2</sub> evolved	Sulfanilamide			Sulapyridine**	Sulfadiazine	Sulfamethyl- diazine	2-aminopyrimi- dine		Sulfathiazole		Sulfamethyl- thiazole	2-Sulfanilamido- 5-ethyl-4-thia- zole		2-aminothiazole	
		0.001M	0.005M	0.02M	0.001M	0.001M	0.001M	0.001M	0.005M	0.001M	0.005M	0.001M	0.001M	0.005M	0.001M	0.005M
<i>Staphylococcus aureus</i>	407*	8	9		0					13	30					
	289*	3			7	7				27	53					
	272*				1					21	56					
	230*									30		25	44		0	15
	199*										78			77		
	234*					12	0	0	2	29	66				8	26
	247*					14	0			27	58				11	21
	402†				3	0		5	4	17	39					
	408†	1	2		25	7	3			24	46	17	48	70		
Mean inhibition .....		5.5	5.0	2.0	7.2	8.0	1.0	2.5	3.0	23.5	53.2	21.0	46.0	73.5	6.3	21.0
<i>Escherichia coli</i>	235*	17	52		8	23				22	55					
	1252†	11	37		2	12				6	34					
	448†	5	33		7	17				15	52					
	773†									15	49	15	59	85	9	14
	767†									8	55	9	48	87	0	10
	1245†				1	0	0	3	7	7	44				0	8
	967†				2	0	3	0	12	37					0	13
Mean inhibition ...		...	11.0	41.0	5.7	11.0	0.0	1.5	1.5	12.0	46.5	12.0	53.5	86.0	2.2	11.2
Baker's yeast	405	33	54		38	25				42	71					
Brewer's yeast	160§	20	58		37	36				43	82					
	180	31	57		24	31				23	63					
	331											48	12			
	335				10		6	12	16	14	51	44	11		5	22
	219													54		
	219													46		
	318				12		0	7	17	10	42				3	12
Mean inhibition .....		...	28.0	56.3	24.2	30.6	3.0	9.5	16.5	26.4	61.6	46.0	11.0	50.0	4.0	17.0
Carboxylase from brewer's yeast	131	21	67		29	41				45	94					
	158	15	57		21	36				30	63					
Mean inhibition .....		...	18.0	62.0	25	38.5				37.5	78.5					

\* Cryochem dried 16 hour cultures.

\*\* In a comparative study the inhibition of the carboxylase activity of *Staphylococcus aureus* at the end of 120 minutes was 50 per cent with sulfathiazole, 15.8 per cent with 2-aminothiazole; no effect was observed with 2-aminopyrimidine or with 2-aminopyridine. The effect on *E. coli* was 46.3, 37.4, 4.1, and 10.1 per cent inhibition respectively. The inhibitory effect on yeast carboxylase activity was 42, 6.4, 7.4, and 3 per cent respectively. The molarity of all drugs was  $5.52 \times 10^{-3}$ .

† Fresh 16 hour cultures.

§ Smaller values obtained with 5 mg of yeast; larger values obtained with 10 mg of yeast.

|| Dialyzed.

preparation from brewer's yeast was studied. In these experiments the optimal conditions of carboxylase activity as described above were employed.

Sparing solubility of some of these substances limited the maximum concentration to  $1.38 \times 10^{-3}$  M. Where it was possible other drugs were compared at a concentration of  $5.52 \times 10^{-3}$  M as well, and sulfanilamide up to  $27.6 \times 10^{-3}$  M. The drugs were dissolved in a saline-phosphate mixture of pH 7.16 (90 cc. saline + 10 cc. M/15 phosphate buffer), heated to 56°C. if necessary.

The reaction system contained, in a volume of 5.8 cc., the drug as indicated in Table III, the suspension of organisms, 0.1 mg. of Mg as magnesium chloride and 26.4 mg. of pyruvic acid (0.0517 M) neutralized to pH 7.1, and a final phosphate buffer concentration of M/125. Pyruvate in a volume of 0.5 cc. was introduced into the system from the side arm after temperature equilibration of the system. Although the optimal carboxylase activity takes place at pH 6.0, a system at pH 7.10 was used in consideration of the greater solubility of the drugs at this pH. The duration of the experiments was 120 minutes. The dry weight of

TABLE IV

<i>Staph. aureus</i> before sulfathiazole treatment			<i>Staph. aureus</i> after sulfathiazole treatment		
Control	Sulfathiazole present		Control*	Sulfathiazole present†	
	$1.38 \times 10^{-3}$ M	$5.52 \times 10^{-3}$ M		$1.38 \times 10^{-3}$ M	$5.52 \times 10^{-3}$ M
c.mm. CO <sub>2</sub> . . . . . 402	332	246	402	335	269

\* Sulfathiazole removed by washing.

† Sulfathiazole removed by washing and added again to the system.

daily samples (per reaction system) of *Staph. aureus* was 4 mg., *E. coli* 3.96 mg., and 5 to 10 mg. of air-dried brewer's yeast. (Table III.)

#### *Removal of Sulfathiazole from Sulfathiazole-Treated Staphylococcus aureus*

In the previous communication it was suggested that the drugs either combine with the enzyme system forming a "drug-protein-coenzyme" complex or displace coenzyme forming a "drug-protein" enzyme analogue.

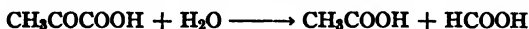
The result of the experiment, given in Table IV, does not give evidence of displacement of cocarboxylase; the drug may possibly have formed a hypothetical reversible "drug-protein-cocarboxylase" complex. A fresh 16 hour culture of *Staph. aureus* was washed with M/15 phosphate buffer of pH 7.16. A dense 2 cc. suspension was treated with 30 cc. of  $5.52 \times 10^{-3}$  M sulfathiazole solution for 35 minutes at 37.5°C. The suspension was then centrifuged and twice washed with phosphate buffer. A control containing no sulfathiazole was given identical treatment. The CO<sub>2</sub> production was then measured in the Barcroft-Warburg set-up.

#### DISCUSSION

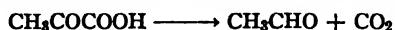
Suspensions of *Streptococcus pyogenes* exercised weak carboxylase activity. The addition of 25–100 γ of cocarboxylase (Merck's) to the reaction system ac-

celerated carboxylase activity several fold. It appears that the carboxylase of the suspension of this organism is impaired in some manner, or perhaps it dissociates more readily than in other cells, and thereby the cocarboxylase is lost.

Barron and Jacobs (8) state that in the absence of oxygen pyruvic acid is split by hemolytic streptococci into acetic acid and formic acid:



Our findings show definitely that the anaerobic metabolism of pyruvic acid involves also its decarboxylation by streptococcal carboxylase:



As far as we know this fact has not been observed previously.

The suspensions of *Pneumococcus* Type 1 did not exercise activity even in the presence of 1–100  $\gamma$  of cocarboxylase. The metabolism of pyruvic acid by pneumococci must therefore take place by a different mechanism. Sevag (2) previously showed that hydrogen peroxide formed from the oxidation of glucose and lactic acid reacts instantaneously with the pyruvic acid resulting simultaneously, and produces acetic acid, carbon dioxide, and water. In the absence of carboxylase in the pneumococcal cells this appears to be the principle mechanism of the decarboxylation of pyruvic acid.

The production of  $\text{CO}_2$  from the anaerobic metabolism of pyruvic acid in *Staph. aureus*, *E. coli*, brewer's yeast, and baker's yeast and a carboxylase preparation from brewer's yeast is inhibited by sulfonamide drugs to varying degrees. The results are given in Table III.

The results clearly show that the thiazole group is the most effective inhibitor of staphylococcal carboxylase; similarly of the *E. coli* carboxylase. Yeast carboxylase and carboxylase prepared from yeast are inhibited by the substances containing pyrimidine, pyridine, and thiazole rings to similar degree, except in sulfamethyldiazine which is completely ineffective on all the organisms studied.

At  $1.38 \times 10^{-3}$  M concentration, 2-aminopyrimidine and 2-aminothiazole show practically no inhibition of *Staph. aureus* and *E. coli* carboxylases. An increase in concentration of 2-aminopyrimidine to  $5.52 \times 10^{-3}$  M shows no increase in inhibition. However, an increase of 2-aminothiazole to  $5.52 \times 10^{-3}$  M concentration results in an inhibition three to five times as great, indicating a definite affinity between this thiazole ring and the enzyme system. With yeast an increase in molarity resulted in an increase in inhibition by both substances. (See also footnote \*\* to Table III.)

Of all the substances tested, 2-sulfanilamido-5-ethyl-4-thiazolone appears to be the most effective inhibitor on *E. coli* and *Staph. aureus*. Its effect on yeast, however, is about equal to that of sulfathiazole. It is also interesting to note that a methyl group on sulfadiazine obviates its inhibitory effect nearly completely, whereas a methyl group on sulfathiazole does not lessen the inhibitory effect on *Staph. aureus* and *E. coli*, and somewhat increases this effect on yeast.



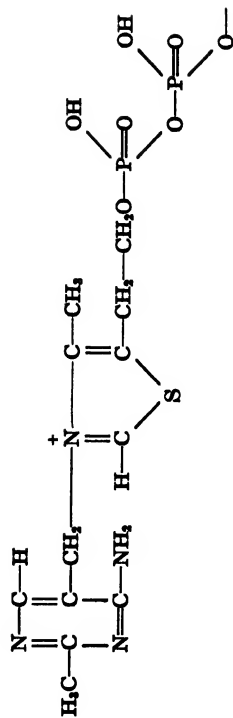
While the inhibiting effect of  $27.6 \times 10^{-3}$  M sulfanilamide on *Staph. aureus* is negligible, it inhibits *E. coli*, yeast, and carboxylase, respectively, 41, 56.3, and 62 per cent. This shows that enzyme proteins in these organisms exhibit different affinities towards this substance.

According to Buchman, Heegard, and Bonner (9) thiazole pyrophosphate markedly inhibits the activity of cocarboxylase. Neither thiazole, nor pyrophosphate alone exercised any inhibiting effect in their experiments. The union between thiazole pyrophosphate and carboxylase protein is stated to be through the pyrophosphate group which is common to both the cocarboxylase and the inhibitor. In our study, we did not find the presence of pyrophosphate group an essential factor in demonstrating the inhibitory effect of the sulfonamides and the thiazole ring on the enzyme system.

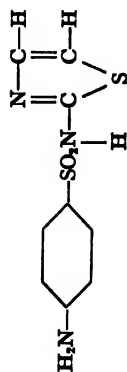
A comparison of the chemical structures of the drugs containing the pyrimidine and thiazole rings with the chemical structure of cocarboxylase containing both rings may help to evaluate the degree of inhibition one can expect them to exercise on the carboxylase system. The results in Table III permit us to state that thiazole groups exercise a high degree of specific inhibition on the *Staph. aureus* and the *E. coli* systems. On the other hand, the carboxylase system of yeast and a carboxylase preparation from yeast are equally affected by sulfonamides containing pyrimidine, thiazole, and pyridine rings; sulfamethyldiazine, however, is completely ineffective on all the organisms studied. Sulfanilamide appears to possess the smallest affinity for the enzyme system. However, it is a potential inhibitor, and this potentiality demonstrates its power when rings such as pyridine, pyrimidine, and thiazole are attached to the sulfamido group.

It is interesting in this connection to refer to the observation made by Rammelkamp and Jewell (10) on the effect of sulfadiazine and sulfathiazole on the growth of *Staph. aureus*. They found that sulfathiazole added to blood exercised a somewhat greater effect than sulfadiazine against staphylococci. Moreover, the action of sulfathiazole becomes manifest at lower concentrations. When the drugs were administered orally, sulfathiazole invariably produced an increase in the bactericidal action of the blood as great or greater than sulfadiazine, even though the concentration of sulfadiazine was twice that of sulfathiazole. Similarly the results obtained by Straus, Dingle, and Finland (11) show that sulfathiazole is about 4 times as effective as sulfadiazine, 8 times as effective as sulfanilamide, and 5-6 times as sulfapyridine on the growth of *Staph. aureus*. Their comparative studies (11) on the inhibition of the growth of *E. coli* with these drugs yielded similar results. In this case, sulfathiazole was 5 times as effective as sulfapyridine, twice as effective as sulfadiazine and 40-50 times as sulfanilamide.

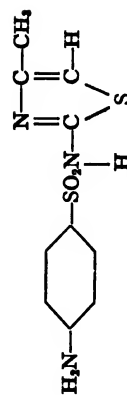
The results given by us in Table III seem to corroborate satisfactorily the findings of the above cited investigators. The relative effectiveness of these



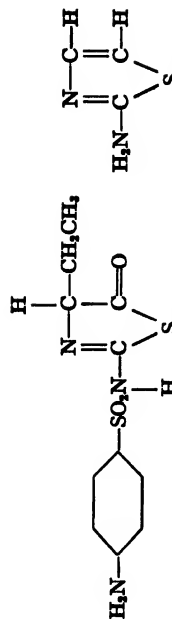
Coccarboxylase



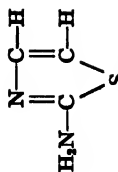
Sulfathiazole;



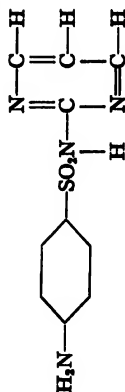
Sulfamethylthiazole;



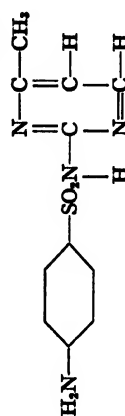
Sulfanilamido-5-ethyl-4-thiazolone;



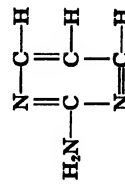
Aminothiazole



Sulfadiazine;



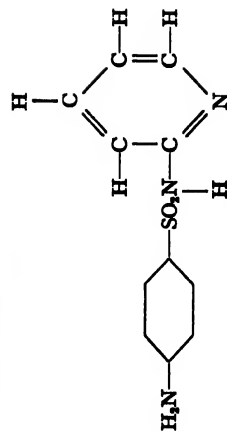
Sulfamethyldiazine;



Aminopyrimidine



Sulfanilamide



Sulfapyridine

drugs on the inhibition of growth and the inhibition of the metabolism of pyruvic acid seems to point to a possible direct relationship, emphasizing the importance of pyruvic acid metabolism during the multiplication of these organisms. On the other hand the failure to find carboxylase in pneumococci suggests that sulfathiazole may inhibit some other enzyme than carboxylase in these microorganisms.

If the sulfonamide drugs exercise their inhibitory effect on enzymes in part by virtue of the similarity of their structure to the coenzyme molecule, as the results presented in this study appear to indicate, then another group of biologically active substances, such as vitamins and vitamin derivatives, structurally related to both the drugs and the coenzymes, might be expected to modify the inhibitory action of the drugs. The present study is being followed by an investigation of this problem.

Since cozymases, like carboxylases, are universally present in the respiratory enzyme system, the possible relation of the action of sulfonamides to the structural similarity existing between them and the cozymases must likewise be investigated. The result of such an investigation will no doubt be significant from the point of view of the theory concerning the mechanism of antibacterial drug action.

The authors wish to express their thanks to Mr. J. Smolens for the nitrogen determinations and to Mr. M. Ibsen for the technical assistance rendered. Thanks are due also to Merck and Company, Dr. M. L. Moore of Sharp & Dohme, and Dr. R. O. Roblin, Jr., of the American Cyanamid Company for some of the substances used in this investigation.

#### SUMMARY

The inhibiting effects of sulfonamide drugs and their derivatives on the anaerobic decarboxylation of pyruvic acid by *Staphylococcus aureus*, *Escherichia coli*, baker's and brewer's yeast, and a carboxylase preparation from brewer's yeast have been investigated. These drugs are: sulfanilamide, sulfapyridine, sulfadiazine, sulfamethyldiazine, sulfathiazole, sulfamethylthiazole, sulfanilamido-5-ethyl-4-thiazolone, 2-aminopyrimidine, 2-aminothiazole, and 2-aminopyridine.

The sulfathiazole ring appears to exercise decidedly greater specific inhibiting effect on the carboxylases of *Staph. aureus* and *E. coli*. The inhibiting effect on yeast carboxylase is non-differentiable among all the substances tried, except sulfamethyldiazine which is completely ineffective on the carboxylases of the organisms studied.

The specific inhibitory effect of sulfathiazole on the carboxylases of *Staph. aureus* and *E. coli* in comparison to sulfanilamide, sulfapyridine, and sulfadiazine is in harmony with *in vivo* and *in vitro* experimental results of other investigators.

The results of the present investigation appear to support the hypothesis (1) that sulfonamides exert their bacteriostatic action through chemical affinity for the carrier proteins of certain respiratory enzymes of the bacterial cell, and that this affinity may in part be related to structural similarity between components of the drugs and the corresponding respiratory coenzymes.

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# ENERGY, QUANTA, AND VISION\*

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## I

### *Threshold Energies for Vision*

The minimum energy required to produce a visual effect achieves its significance by virtue of the quantum nature of light. Like all radiation, light is emitted and absorbed in discrete units or quanta, whose energy content is equal to its frequency  $\nu$  multiplied by Planck's constant  $h$ . At the threshold of vision these quanta are used for the photodecomposition of visual purple, and in conformity with Einstein's equivalence law each absorbed quantum transforms one molecule of visual purple (Dartnall, Goodeve, and Lythgoe, 1938). Since even the earliest measurements show that only a small number of quanta is required for a threshold stimulus, it follows that only a small number of primary molecular transformations is enough to supply the initial impetus for a visual act. The precise number of these molecular changes becomes of obvious importance in understanding the visual receptor process, and it is this which has led us to the present investigation.

The first measurements of the energy at the visual threshold were made by Langley (1889) with the bolometer he invented for such purposes (Langley, 1881). He found the energy to be  $3 \times 10^{-9}$  ergs for light of  $550 \mu$ . Langley worked before the physiology of vision was understood, so that he used the wrong light and took none of the precautions now known to be necessary; even so, his results are too high only by a factor of 10.

In the fifty years since Langley there have been eleven efforts to redetermine the minimum energy for vision. We have carefully studied all these accounts and have done our best to evaluate the measurements. Unfortunately, many of them contain serious errors which invalidate them. Most of them involved no direct energy determinations; instead, the investigators relied on previously measured energy distributions in standard sources and made elaborate computations from them. Only a few can be considered as reliable.

After Langley, the earliest paper is by Grijns and Noyons (1905). Their data differ widely from all other measurements and cannot be accepted even

\* A preliminary report of these measurements was published in *Science* (Hecht, Schlaer, and Pirenne, 1941), and presented to the Optical Society in October, 1941 (Hecht, 1942).

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though it is hard to discover their precise errors because the description is too obscure. Zwaardemaker (1905), in whose laboratory their measurements were made, reports some of his own rough determinations, which turn out to be near Langley's. Neither Grijns and Noyons nor Zwaardemaker actually measured the energies involved, but relied on Ångström's (1903) determinations of the energy distribution in the Hefner lamp.

The best of the early efforts is by von Kries and Eyster (1907); and though the results involve many calculations, they come very close to the most careful of modern measurements. Von Kries and Eyster made no direct energy determinations; they measured brightnesses, durations, and areas. The conversion of these factors into final energies requires skill and care in the evaluation of absorptions, reflections, lens factors, and the like, and it is gratifying to see the admirable way in which von Kries accomplished this task.

TABLE I  
*Minimum Energy for Vision*

Wavelength	Energy	No. of quanta	Source
$m\mu$	$ergs$		
505	$0.66-1.17 \times 10^{-10}$	17-30*	Chariton and Lea (1929)
507	$1.3-2.6 \times 10^{-10}$	34-68	von Kries and Eyster (1907)
530	$1.5-3.3 \times 10^{-10}$	40-90	Barnes and Czerny (1932)

\* For inexperienced observers.

Computations from star magnitudes were made by Ives (1916) and by Russell (1917). However, neither they nor Reeves (1917) and Buisson (1917), who both reproduced star observations in the laboratory, employed the best physiological conditions for the measurements. Moreover, none of them took consideration of the different luminosity curves for rod vision and cone vision, and used the latter as standard in the computations.

Direct energy measurements were made by du Noüy (1921), but his work involves serious physical errors, and his results are too low by a factor of more than 100—so low indeed as to seem impossible.

The most recent determinations are by Chariton and Lea (1929), by Wentworth (1930), and by Barnes and Czerny (1932), all of whom agree in the order of magnitude of their results. Wentworth's exposures were too long to yield minimal values; otherwise her work is excellent. She measured the energies involved, which Barnes and Czerny also did, but not as directly.

From these twelve researches, we have chosen the three sets of measurements which are free from what can now be recognized as obvious error. These are given in Table I. Even though they differ by a factor of about 3, these data can be considered as roughly confirming one another. However,

since for our purposes a factor of 3 cannot be ignored, we undertook to make the measurements again, but under the best physical and physiological conditions.

## II

### *Visual Conditions*

The circumstances which will yield the maximum retinal sensibility have been adequately known for years. They involve dark adaptation, peripheral vision, small test fields, short exposures, and selected portions of the spectrum.

Complete dark adaptation means a stay of at least 30 minutes in the dark before measurements can be begun (Piper, 1903; Hecht, Haig, and Chase, 1937). After thorough dark adaptation the periphery of the retina is much more sensitive than its center. The greatest density of rod elements begins at about  $18^\circ$  out (Østerberg, 1935), and exploration shows that between  $20^\circ$  and  $30^\circ$  from the center there is a region of maximum sensibility to light (Wentworth, 1930). The variation within this region is not large, and for convenience we chose a retinal area situated  $20^\circ$  temporally on the horizontal axis.

In visual threshold measurements it has been established that the larger the test area, the smaller need the intensity be for its recognition (*cf.* summary by Wald, 1938 *a*). This reciprocal relation is exact only for small areas. Our preliminary experiments, as well as the work of other investigators, show a minimum for the product of area and intensity for fields of the order of 10 minutes diameter. We therefore chose a circular retinal area of 10 minutes diameter for the test field.

The energy required to pass over the visual threshold involves an approximately reciprocal relationship between intensity and time of exposure. For exposures shorter than 0.01 second, the reciprocal relation holds perfectly (Graham and Margaria, 1935). To be sure of falling within this most efficient range, our exposures were 0.001 second long.

Finally, from the measurements of the scotopic luminosity curve (Hecht and Williams, 1922), it is known that for dim vision the eye is most sensitive to a wavelength of  $510\text{ m}\mu$ , and this is the light which we used for making the measurements.

## III

### *Apparatus and Calibrations*

The physical arrangements may be seen in Fig. 1. The light source  $L$  is a ribbon filament lamp run on constant current obtained from storage cells and measured potentiometrically. By means of a lens, it is focussed on the slit of a double monochromator  $M_1M_2$  and finally on the artificial pupil  $P$ . The subject, who sits in a dark cabinet in the dark room, has his head in a fixed position by keeping his teeth in a "bite" or hard impression of his upper jaw. He has his left eye next to the pupil  $P$ , and on looking at the red fixation point  $FP$  he sees the field lens  $FL$ . The light intensity of this



uniformly illuminated field is varied in large steps by the neutral filters  $F$ , and in a gradual way by the neutral wedge and balancer  $W$ . The size of the field is controlled by the diaphragms  $D$ . Its exposure is fixed by the shutter  $S$ , and is initiated by the subject.

For the record it is necessary to describe the apparatus and calibrations in detail. The double monochromator is made of two individual constant deviation monochromators,  $M_1$  and  $M_2$ , which are arranged for zero dispersion by means of the reversing prism  $RP$ . In this way, all the light passes through an equal thickness of glass, and assures a uniform brightness of the field lens  $FL$ . The exit slit of  $M_1$  has been removed, and the entrance slit of  $M_2$  serves as the middle slit of the combined double monochromator. The entrance and exit slits of the combination are kept at 1.2 mm., which corresponds to a band width of  $10\text{ m}\mu$  centered at  $510\text{ m}\mu$ . The middle slit, before which the shutter is placed, is kept at 0.1 mm.

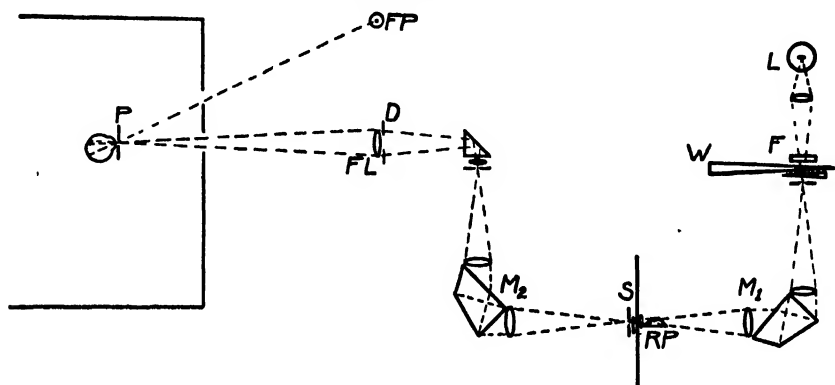


FIG. 1. Optical system for measuring minimum energies necessary for vision. The eye at the pupil  $P$  fixates the red point  $FP$  and observes the test field formed by the lens  $FL$  and the diaphragm  $D$ . The light for this field comes from the lamp  $L$  through the neutral filter  $F$  and wedge  $W$ , through the double monochromator  $M_1M_2$  and is controlled by the shutter  $S$ .

The field lens  $FL$  magnifies the exit slit by a factor of 2, and thus yields an image of it 2.4 mm. wide and over 10 mm. high at the pupil  $P$ . The image is sufficient to cover uniformly not only the pupil  $P$ , but also the linear thermopile used for the energy calibration. The pupil mount at  $P$  and the field lens  $FL$  are connected by a carefully diaphragmed and blackened tube. The 2 mm. circular pupil  $P$  used for the visual measurements can be replaced by a slit 2 mm. wide and 10 mm. high behind which is the receiving surface of the thermopile for energy measurements.

$S$  is a precision shutter made of two parts. One part is a thin circular aluminum disc with a small sector of  $10.8^\circ$  removed and properly balanced. It is run at 1800 R.P.M. by means of a synchronous motor, and therefore permits light to pass through the middle slit for  $1/1000$  second during each revolution. The other part is a polar relay shutter, which, by means of a phasing commutator on the shaft of the synchronous motor, is opened for only one passage of the rotating disc aperture whenever the subject releases a push button.

The essentials of the shutter are shown in diagrammatic detail in Fig. 2. On the same shaft with the disc there is mounted a commutator having a "live" sector, which together with the brush occupies somewhat less than  $90^\circ$ . Two brushes are arranged on this commutator  $90^\circ$  apart, and are so phased with the A.C. line voltage that one of these brushes receives only a positive impulse while the other receives only a negative impulse. These impulses control a polar relay  $PR_2$ , which then actuates a pair of single pole, double throw micro switches,  $MS_1$  and  $MS_2$ . These are arranged with their springs in opposition in such a manner that the switches are in equilibrium

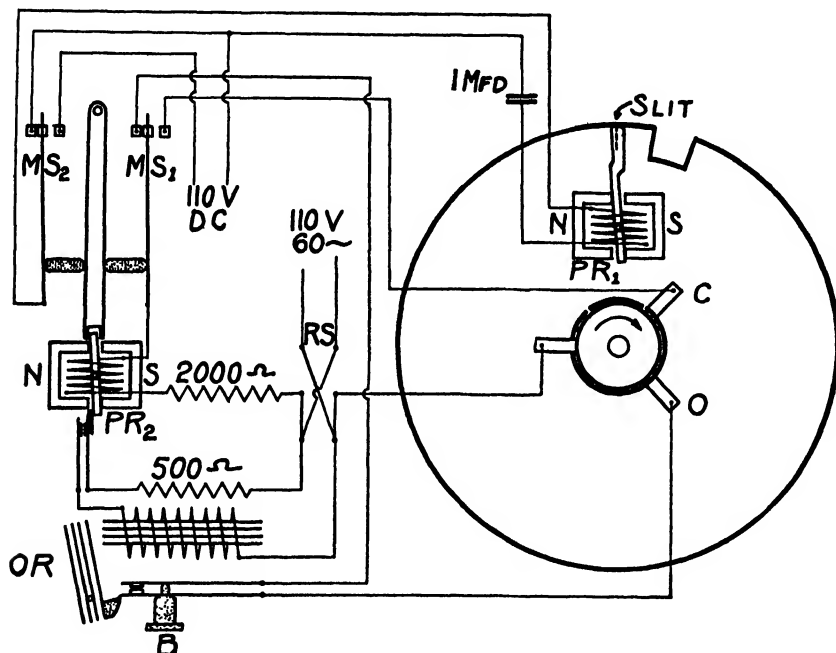


FIG. 2. Shutter for obtaining a single exposure of  $1/1000$  second. The details are described in the text.

at either of their two positions, and require but a small force and movement to kick them over to their other positions. Micro switch  $MS_1$  is in series with the winding of  $PR_2$ , and in one position connects with the opening brush  $O$  and in the other position with closing brush  $C$ . The other micro switch,  $MS_2$ , charges and discharges a  $1 \mu\text{fd}$ . condenser from the 110 volt, d.c. line through the polar relay  $PR_1$ . These impulses in and out of the condenser actuate  $PR_1$  whose armature movement then uncovers and covers the middle slit.

The operation is seen by following a single cycle of operation of circuit and shutter. Fig. 2 shows the apparatus during its rest or closed period. The 110 volt, 60 cycle power enters through a pole-reversing switch,  $RS$ , to the neutral brush on the commutator. The impulse through the closing brush  $C$  is blocked, since it enters an open

contact in  $MS_1$ . The impulse going to the opening brush  $O$  is blocked at the secondary contacts of the overload relay  $OR$ , the push button of which,  $B$ , is controlled by the observer. When  $B$  is released so that contact is made, the next impulse which leaves the opening brush  $O$  goes through the left hand contact of  $MS_1$ , through the winding of  $PR_2$ , and through a 2,000 ohm resistance to the other side of the power line. This impulse through  $PR_2$  is adequate to throw its armature to the other position, thus switching over both  $MS_1$  and  $MS_2$ , and closing the power circuit through the primary of the overload relay  $OR$ . The activation of  $OR$  closes its armature, whose movement opens the secondary contacts attached to it, thereby breaking the circuit from the opening brush  $O$  so that the cycle does not repeat itself. The switching of  $MS_2$  to its right contact charges the 1  $\mu$ fd. condenser through  $PR_1$ , which moves its armature and thereby exposes the slit. The switching of  $MS_1$  to its right contact sets the circuit for the very next impulse through the closing brush  $C$  to  $PR_2$ . This closing impulse comes exactly three half-cycles or 3/120 second after the original opening impulse, and causes  $PR_2$  to return to its original position. Now  $MS_2$  discharges the 1  $\mu$ fd. condenser, which actuates  $PR_1$  so that its armature moves to cover the slit and terminate the cycle.

The pole-reversing switch  $RS$  enables one to select the correct polarity for the operation of this circuit. It needs to be set only at the beginning of an experiment when the synchronous motor is first started.

$PR_1$  and  $PR_2$  are old Baldwin speaker units in which all the spring tension restraint of the armature has been removed; they thus act as very fast polar relays. An oscillographic study of  $PR_2$ , which is essentially unloaded, shows that the micro switches are thrown to the right contacts before the end of the half cycle which actuates it. However,  $PR_1$ , due to the loading of the shutter vane attached to the armature, is not nearly so fast, but opens in less than 3/120 second and closes in less than 4/120 second, which are the limits required for its operation.  $MS_1$  and  $MS_2$  are a pair of micro switches, type Z,—BZ-R, selected for near equality of spring tension. They are mounted plunger to plunger with a loose bar between them. This bar has a fulcrum at one end, and a fork at the other. Inside the fork is located the armature of  $PR_2$ . The fork width is so adjusted that it offers no resistance to the movement of the armature except at the very end of its motion when the impact of the armature is sufficient to kick over both micro switches.

It was necessary to calibrate the neutral filters, the wedge and balancer, the diaphragm openings, and the energy at the pupil  $P$ . The filters and the wedge and balancer were measured with our photoelectric spectrophotometer (Shlaer, 1938) at the same wavelength used in the experiments, and in an analogous optical position in front of the entrance slit of the first monochromator. We first used filters and wedges made of gelatin; later they were replaced with neutral glass. The smaller diaphragms were calibrated under the microscope with a filar micrometer by measuring several diameters for each opening; the larger ones were similarly measured with a comparator.

The energy density at the pupil  $P$  was measured with a Hilger linear thermopile and a Paschen galvanometer. The thermopile was first standardized against a standard carbon filament lamp of known energy radiation. To do this we used the tube holding the pupil and the field lens, first removing the field lens and substituting

the slit for the pupil, and fixing the thermopile immediately behind the slit. This assembly of tube, slit, and thermopile was then mounted on an optical bench so that the standard lamp was at the specified distance of 2 meters from the receiver of the thermopile. The thermopile and its end of the tube was then covered with a thermos flask and allowed to reach thermal equilibrium. Between the source and the opening of the tube was mounted a triple leafed shutter with about 20 cm. spacing between the leaves. The surfaces facing the thermopile were blackened while those facing the source were shiny. This shutter was used to open and close the radiation to the thermopile.

The thermopile was connected to a Paschen galvanometer, which is a moving magnet type of very high sensitivity (about  $2 \times 10^{-9}$  volts per mm. at a meter). In series with the thermopile and galvanometer was a resistance of about 0.1 ohm, across which known potentials could be inserted to counterbalance the potential generated by the thermopile, thus using the galvanometer as a null point instrument. The radiation was first permitted to fall on the thermopile, and the galvanometer brought back to zero by means of measured counter-potentials. The radiation was then occluded and the counter-potential switched off to check the zero of the galvanometer. In this way we could measure large potentials corresponding to galvanometer swings of several meters without actually using such scale distances. The thermopile was calibrated as potential *vs.* radiant energy density incident upon its receivers for three different energy densities which covered a range of about 3 to 1, and included the actual energy density delivered by the ribbon filament lamp and the monochromators.

For calibrating the energy density through the monochromators, the field lens was replaced in the tube and the tube placed in its correct position in the apparatus. Diaphragm *D* was removed, the middle slit of the monochromator was opened to 1.5 mm., and the wedge and balancers were removed. The energy was then measured with the same thermopile and the same electrical system. With the lamp current at 19 amperes, the energy density at the pupil *P* was 27.5 microwatts per square centimeter; with the current at 18 amperes, it was 18.3 microwatts per square centimeter. In the early visual determinations we used the lamp at 19 amperes; in the later determinations at 18 amperes.

In order to convert these measurements into values of the energy at the pupil during the visual determinations, it is necessary to reduce the measured energy density by factors corresponding (a) to the change of the middle slit from 1.5 to 0.1 mm., (b) to the change in aperture of the field lens from its largest opening of 25.9 mm. diameter to the sizes of the particular diaphragms used, and (c) to the insertion of the wedge and balancer. All these factors were known from previous separate measurements, but we calibrated them again in their places in the apparatus by means of a sensitive dry-disc photocell in place of the thermopile behind the thermopile slit. The results merely confirmed the previous calibrations. By applying these reduction factors for the wedge at its thinnest place, the middle slit at 0.1 mm., the 10 minute diaphragm at the field lens, and the 2 mm. pupil at *P*, we found that the energy density through the pupil is  $3.4 \times 10^{-4}$  ergs per second when the ribbon filament lamp is running at 18 amperes. The energy calibrations were run through twice several months apart and agreed almost perfectly.

## IV

*Visual Measurements*

From the subject's point of view, an experiment involves the report of whether or not he has seen a flash of light after he has opened the shutter for an exposure. Fixation of the red point need not be continuous, a circumstance which avoids undue fatigue. The observer is told by the operator that conditions are set and that he should try a flash when he is ready. He fixates

TABLE II

*Minimum Energy for Vision*

Each datum is the result of many measurements during a single experimental period, and is the energy which can be seen with 60 per cent frequency.  $\lambda = 510 \text{ m}\mu$ ;  $h\nu = 3.84 \times 10^{-12}$  ergs.

Observer	Energy	No. of quanta	Observer	Energy	No. of quanta
	<i>ergs <math>\times 10^{10}</math></i>			<i>ergs <math>\times 10^{10}</math></i>	
S. H.	4.83	126	C. D. H.	2.50	65
	5.18	135		2.92	76
	4.11	107		2.23	58
	3.34	87		2.23	58
	3.03	79	M. S.	3.31	81
	4.72	123		4.30	112
	5.68	148			
S. S.	3.03	79	S. R. F.	4.61	120
	2.07	54	A. F. B.	3.19	83
	2.15	56			
	2.38	62	M. H. P.	3.03	79
	3.69	96		3.19	83
	3.80	99		5.30	138
	3.99	104			

the red point, and at the moment which he considers propitious, he exposes the light to his eye. The operator changes the position of the wedge, or removes or introduces a filter until he is satisfied with the precision of the measurements.

In the early measurements we considered that the threshold had been reached when the observer saw a flash of light at a given intensity six times out of ten presentations. Later the measurements were made somewhat more elaborately. Each of a series of intensities was presented many times and the frequency of seeing the flash was determined for each. From the resulting plot of frequency against intensity we chose the threshold as that amount of light which could be seen with a frequency of 60 per cent.

During 1940 and 1941 we measured the threshold for seven subjects. With

four we made several determinations each, extending over a year and a half; one subject we measured on two occasions 3 months apart; and two we measured only once. For all these observers the minimum energy necessary for vision ranges between 2.1 and  $5.7 \times 10^{-10}$  ergs at the cornea. These small energies represent between 54 and 148 quanta of blue-green light. The results for the individual subjects are in Table II, and are given as energy and as the number of quanta required.

It is to be noticed that these values are of the same order of magnitude as those of von Kries and Eyster, and of Barnes and Czerny, but almost twice as large. Because of the fairly wide ranges, these previous measurements and our own overlap to some extent, and it is conceivable, though not probable, that their observers may actually have needed somewhat smaller energies than ours. Chariton and Lea's results, however, are much too small. Actually their value of  $17 h\nu$  is an extrapolation to zero frequency of seeing; if we take as threshold a 60 per cent frequency, their data come more nearly to  $25 h\nu$ . This is still too small a value, and is probably in error, as will be apparent in later sections of our paper.

## V

### *Reflections and Absorptions*

The values in Table II, as well as those of previous investigators, are the energies incident at the cornea. Nevertheless the tacit supposition has generally been made that they represent the actual energies necessary to initiate a visual act. It is important to recognize that this assumption is incorrect. Before one can know how many quanta are required to start the visual process, one must apply at least three corrections to the measurements.

The first is reflection from the cornea. This is about 4 per cent and is obviously of not much importance. The second involves loss by the ocular media between the outer surface of the cornea and the retina. It has been common opinion that this loss is small. However, the measurements of Roggenbau and Wetthauer (1927) on cattle eyes, as well as the recent measurements of Ludvigh and McCarthy (1938) on human eyes, have shown that this loss is large. From the values of Ludvigh and McCarthy it appears that at  $510 \mu$  the ocular media transmit almost exactly 50 per cent of the light entering the cornea of a young person, and less of an older one.

The next correction is much more difficult to evaluate with precision and involves the percentage of the energy absorbed by the retinal elements themselves. Since visual purple is the photosensitive substance concerned in this particular act, light which is not absorbed by it is visually useless. One cannot assume that visual purple absorbs all the light incident on the retinal cells. The fraction which it does absorb must be found by experiment.

Koenig (1894) determined the absorption of the total amount of visual

purple which can be extracted from the human eye. If this amount of visual purple is spread evenly over the whole retina, his data show that it will absorb only 4 per cent of light of 510  $m\mu$ . This is a small value. Nevertheless, it is about the same as the 4 per cent and the 13 per cent recently found by Wald (1938 *b*) with a similar method for the absorption of the visual purple of the rabbit and rat retinas respectively.

These figures are probably too low, first because it is unlikely that all of the visual purple in the eye has been extracted, and second, because visual purple is not evenly distributed over the retina. It is lacking in the fovea; and even in the periphery the density of the rods is known to vary in a definite way. However, these absorptions may be considered as lower limiting values.

## VI

### *Visual Purple Absorption*

We have estimated the absorption of visual purple in the retina in a completely independent manner by comparing the percentage absorption spectrum of different concentrations of visual purple with the scotopic (rod) luminosity curve of the eye measured at the retina. The comparison rests on the fact that the shape and width of the percentage absorption spectrum of a substance varies with its concentration, and that the luminosity curve must represent the percentage absorption curve of a particular concentration of visual purple in the retina.

Fig. 3 shows the absorption spectrum of frog's visual purple as determined by Chase and Haig (1938) in our laboratory, by Lythgoe (1937) in London, and by Wald (1938 *b*) at Harvard. The agreement of the data is obvious, and shows that the absorption spectrum of visual purple may be considered as well established. Table III gives the average of these three series of measurements computed so that the maximum density at 500  $m\mu$  has a value of 1.

From these data in Table III we may prepare a series of percentage absorption spectra for different concentrations of visual purple. Since we are not interested in the absolute concentration of visual purple, but rather in its absorption capacities, we can deal with the series of percentage absorption spectra entirely in terms of maximum absorption. It will be recalled that the photometric density  $d$  is related to the transmission  $I_t$  by the equation  $d = \log(1/I_t)$ , and since the absorption  $I_a = 1 - I_t$ , it is a simple computation to find the percentage absorption corresponding to any density value, or the reverse.

We have made such computations for a variety of visual purple densities, and Fig. 4 shows the resulting percentage absorption curves for the different maximal absorptions of visual purple. For comparisons among the curves in Fig. 4 the maxima have all been made equal to 1, but their actual values are

indicated in the figure. It is clear that the width of the curves increases as the concentration of visual purple increases.

The scotopic luminosity curve, as measured experimentally, records the reciprocal of the relative energy in different parts of the spectrum required for the production of a constant and very low brightness in the eye (Hecht and Williams, 1922). If this is to be compared with the absorption spectrum of

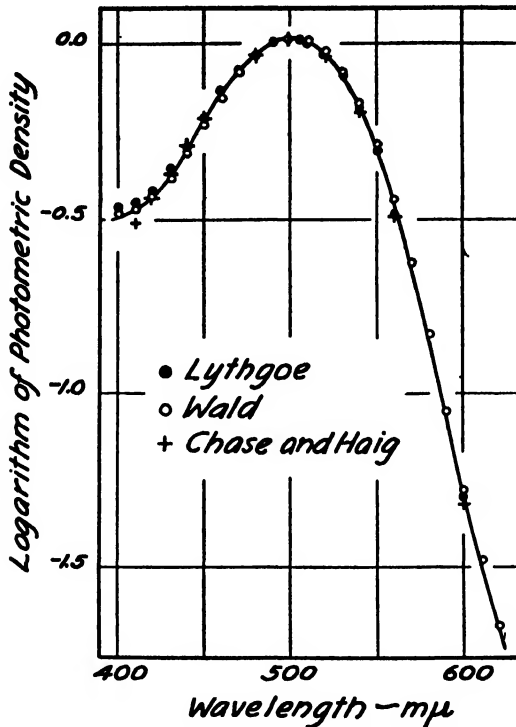


FIG. 3. Absorption spectrum of frog's visual purple. The data from the three sources have been made equal at 500 mμ.

visual purple, it must be converted into a quantum luminosity curve instead of an energy luminosity curve, because it is the number of quanta which determines the photochemical effectiveness of light and not just its energy content (Dartnall and Goodeve, 1937). Moreover, since our interest lies in retinal comparisons, the luminosity curve must be corrected for ocular media absorption in terms of the data of Ludvig and McCarthy.

The scotopic luminosity data have been corrected in these two ways; the computed values are given in Table IV and shown as circles in Fig. 5. Included in the same figure are two percentage absorption spectra of visual purple; the



upper curve represents 20 per cent maximal absorption, while the lower curve is 5 per cent maximal absorption.

TABLE III  
*Absorption Spectrum of Visual Purple*

Average of data from Chase and Haig (1938), Wald (1938 b), and Lythgoe (1937).

$\lambda - m\mu$	Density	$\lambda - m\mu$	Density	$\lambda - m\mu$	Density
400	0.306	480	0.900	560	0.321
410	0.317	490	0.967	570	0.207
420	0.353	500	1.000	580	0.131
430	0.408	510	0.973	590	0.0805
440	0.485	520	0.900	600	0.0473
450	0.581	530	0.780	610	0.0269
460	0.691	540	0.628	620	0.0150
470	0.811	550	0.465		

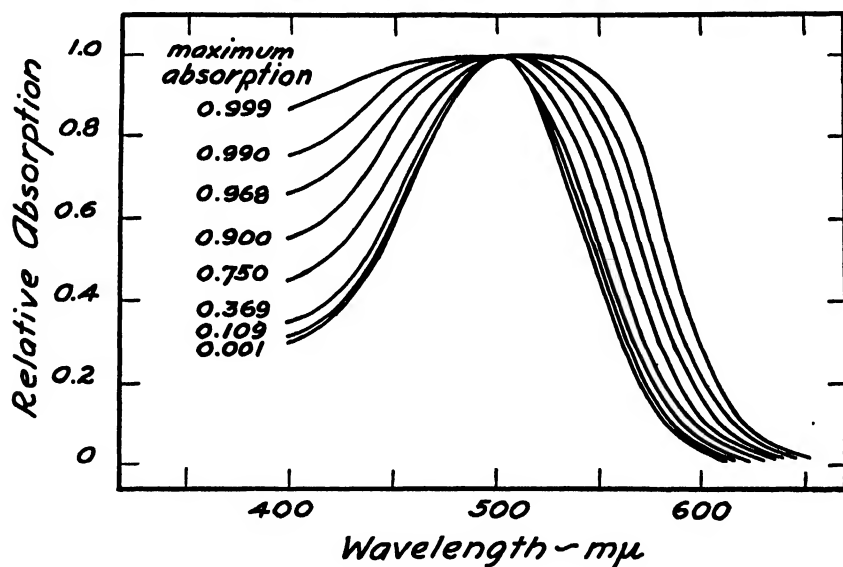


FIG. 4. Percentage absorption spectra of various concentrations of visual purple. For convenience in comparing the shapes of the curves, their maxima have all been equated to 1 and superimposed. The actual fraction absorbed at the maximum is shown for each curve. It is apparent that with increasing concentration the absorption curve steadily increases in width.

For comparing the luminosity and absorption data, it is well to confine our attention mostly to the long wave half of the luminosity curve because of the larger number of points involved. From the comparison it is apparent that the 5 per cent maximum absorption curve describes the points quite well, but

TABLE IV

*Rod Luminosity Distribution in Spectrum*

The original energy luminosity data of Hecht and Williams (1922) in column 2, when divided by the corresponding wavelengths in column 1, yield the quantum luminosity values in column 3 after being multiplied by a factor so that the maximum at 511  $m\mu$  equals 1. When these values in column 3 are divided by the ocular media transmission data in column 4 from Ludvigh and McCarthy (1938), they yield the spectral luminosity distribution at the retina given in column 5 after multiplication by a factor so that the maximum at 502  $m\mu$  is 1.

$\lambda - m\mu$	Energy luminosity at cornea	Quantum luminosity at cornea	Ocular transmission	Quantum luminosity at retina
412	0.0632	0.0779	0.116	0.336
455	0.399	0.447	0.410	0.545
486	0.834	0.874	0.472	0.926
496	0.939	0.964	0.490	0.984
507	0.993	0.998	0.506	0.986
518	0.973	0.957	0.519	0.921
529	0.911	0.877	0.540	0.812
540	0.788	0.743	0.559	0.665
550	0.556	0.515	0.566	0.455
582	0.178	0.155	0.596	0.131
613	0.0272	0.0226	0.625	0.0181
666	0.00181	0.00139	0.672	0.00104

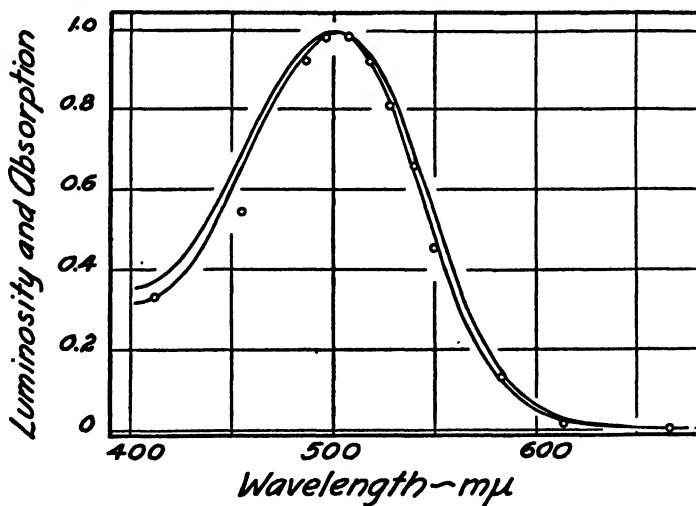


FIG. 5. Comparison of scotopic luminosity at the retina with visual purple absorption. The points are the data of Hecht and Williams corrected for quantum effectiveness and ocular media transmission. The curves are the percentage absorption spectra of visual purple; the upper curve represents 20 per cent maximal absorption, and the lower one 5 per cent maximal absorption. All curves have been made equal to 1 at the maximum, 500  $m\mu$ , for ease in comparison.

that the 20 per cent curve is definitely excluded, because its absorption on both sides is just too high. The 10 per cent absorption curve, not shown in the figure, is perhaps slightly better than the 5 per cent one; it cuts through more points. In any case, both values are of the same order of magnitude as those found by Koenig and by Wald. However, to be quite safe, we may take 20 per cent as the upper limit for the absorption of 510 m $\mu$  by the visual purple in the human retina after complete dark adaptation.

## VII

### *Energy Absorbed by the Rods*

It is clear now why the 54 to 148 quanta required at the cornea cannot represent the energy actually employed in vision. About 4 per cent of this incident light is reflected by the cornea; almost precisely 50 per cent is absorbed by the lens and other ocular media; and of the rest, at least 80 per cent passes through the retina without being absorbed. If corrections are made for these factors, the range of 54 to 148 quanta at the cornea becomes as an upper limit 5 to 14 quanta absorbed by the visual purple of the retina.

Visual purple is in the terminal segments of the rods, and the 10 minute circular visual field contains about 500 rods (Østerberg, 1935). Since the number of absorbed quanta is so small, it is very unlikely that any one rod will take up more than one quantum. In fact, the simplest statistical considerations show that if 7 quanta are absorbed by 500 rods, there is only a 4 per cent probability that 2 quanta will be taken up by a single rod. We may therefore conclude that in order for us to see, it is necessary for only 1 quantum of light to be absorbed by each of 5 to 14 retinal rods.<sup>1</sup>

It is very likely that the photodecomposition of visual purple in solution has a quantum efficiency of 1 (Dartnall, Goodeve, and Lythgoe, 1938). Our data then mean that 1 molecule of visual purple needs to be changed simultaneously in each of 5 to 14 rods, in order to produce a visual effect. This is indeed a small number of chemical events, but by virtue of its very smallness, its reality may be tested in an entirely independent manner.

## VIII

### *Poisson Distributions*

The energy calibration of the light gives merely the average number of quanta per flash. This is in the nature of the measurement, because the

<sup>1</sup> These data disprove the supposition made by Granit, Holmberg, and Zewi (1938) that most of the visual purple in the retina is inert as sensory substance, and that sensory impulses from the rods are "initiated by the bleaching of a thin surface film, which had to contain only an immeasurably small fraction of the total quantity present" (Granit, Munsterhjelm, and Zewi, 1939). Since the maximum visual purple concentration which the retina can achieve is able to absorb only 5 to 14 quanta at the threshold of vision, a very small fraction of the total visual purple would absorb much less than one quantum and would be ineffective for visual purposes.

thermopile records only the energy density, which is the number of quanta per second from a continuously incident light. Each flash, however, will not always deliver this average number. Sometimes the flash will yield fewer, sometimes more, quanta.

Since absorption of this group of quanta by the retina represents discrete and independent events which occur individually and collectively at random, the actual number of such retinal events which any given flash provides will vary according to a Poisson probability distribution (Fry, 1928). Let  $n$  be the number of quanta which it is necessary for the retina to absorb in order for us

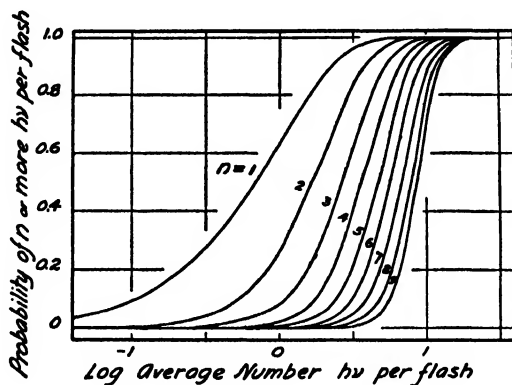


FIG. 6. Poisson probability distributions. For any average number of quanta ( $h\nu$ ) per flash, the ordinates give the probabilities that the flash will deliver to the retina  $n$  or more quanta, depending on the value assumed for  $n$ .

to see a flash of light. Let  $a$  be the average number of quanta which any flash yields to the retina. Then the Poisson distribution states that

$$P_n = a^n / e^n n!$$

in which  $P_n$  is the probability that the flash will yield the necessary  $n$  quanta, and  $e$  is the base of natural logarithms. A special virtue of the Poisson distribution is that it has only one parameter, and is thus determined when the average number  $a$  is set. The values of  $P_n$  for various values of  $a$  and  $n$  are available in printed tables (e.g. Fry, 1928).

Since for us to see a flash of light the retina must absorb  $n$  quanta, we shall also see when the retina absorbs more than  $n$  quanta. From the published Poisson distributions, one can then compute the probability that  $n$  or more quanta will be delivered to the retina in a given flash when the average number of quanta delivered by that flash is known. The values computed in this way for different values of  $a$  and  $n$  are shown in Fig. 6.

There are two significant features of Fig. 6. One is that the shape of the distributions is fixed and different for every value of  $n$ . The curve becomes

steeper as  $n$  increases. It follows from this that if the probability distribution could be determined by experiment, its shape would automatically reveal the value of  $n$  corresponding to it.

Another and equally important feature of Fig. 6 is that the relationship is expressed in terms of the logarithm of the average number of quanta per flash. Therefore, for comparison with the distributions in Fig. 6, the experiments need not employ the absolute values of the average number of quanta delivered per flash, but merely their relative values.

The experiments may then be made quite simply. On many repetitions of a flash of given average energy content, the frequency with which the flash is seen will depend on the probability with which it yields  $n$  or more quanta to the retina. When this frequency is measured for each of several intensities, a distribution is secured whose shape, when plotted against the logarithm of the average energy content, should correspond to one of the probability distributions in Fig. 6, and should thus show what the value of  $n$  has been.

## IX

### *Frequency of Seeing*

We have made determinations of this kind. The experimenter varies the intensity of the light by placing the wedge in specific positions unknown to the observer. The observer then elicits the flash whenever he is ready, and merely reports whether he has seen it or not. The intensities are presented in a deliberately random sequence, each for a specific number of times, usually 50. The procedure is simplified for the operator by a series of accurately made stops against which the wedge may be rapidly set in predetermined positions. A complete series in which six intensities are used requires about  $1\frac{1}{2}$  hours of continuous experimentation composed of two or three periods of intensive work.

The comfort of the observer is of great importance and this must be at a maximum. It is equally important that fixation should not be rigidly continuous because this is fatiguing. Above all, the observer must be on guard to record any subjective feelings of fatigue the moment they become apparent. The experiment is much facilitated by the fact that the observer controls the occurrence of the flash, and can set it off only when he is thoroughly fixated and ready for an observation.

The data for the three observers who engaged in this experiment are given in Table V. One experiment for each observer is plotted in Fig. 7. The points in the figure record the percentage frequency with which a flash of light is seen for flashes of average quantum content shown in the abscissas. Comparison with the curves in Fig. 6 shows that the measurements are best fitted by Poisson distributions in which  $n$  is 5, 6, and 7 quanta per flash. For the two other

experiments in Table IV,  $n$  is 7 and 8. No special statistical methods are necessary to determine which curve fits the data, since smaller and larger values of  $n$  are easily excluded by the simplest visual comparison.

TABLE V

*Energy and Frequency of Seeing*

Relation between the average number of quanta per flash at the cornea and the frequency with which the flash is seen. Each frequency represents 50 flashes, except for S. H. for whom there were 35 and 40 for the first and second series respectively.

S. H.		S. H.		S. S.		S. S.		M. H. P.	
No. of quanta	Frequency	No. of quanta	Frequency	No. of quanta	Frequency	No. of quanta	Frequency	No. of quanta	Frequency
	<i>per cent</i>		<i>per cent</i>		<i>per cent</i>		<i>per cent</i>		<i>per cent</i>
46.9	0.0	37.1	0.0	24.1	0.0	23.5	0.0	37.6	6.0
73.1	9.4	58.5	7.5	37.6	4.0	37.1	0.0	58.6	6.0
113.8	33.3	92.9	40.0	58.6	18.0	58.5	12.0	91.0	24.0
177.4	73.5	148.6	80.0	91.0	54.0	92.9	44.0	141.9	66.0
276.1	100.0	239.3	97.5	141.9	94.0	148.6	94.0	221.3	88.0
421.7	100.0	386.4	100.0	221.3	100.0	239.3	100.0	342.8	100.0

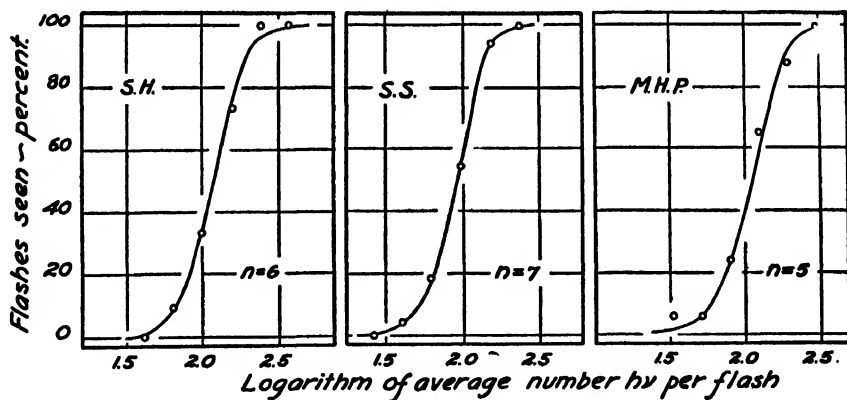


FIG. 7. Relation between the average energy content of a flash of light (in number of  $h\nu$ ) and the frequency with which it is seen by three observers. Each point represents 50 flashes, except for S.H. where the number is 35. The curves are the Poisson distributions of Fig. 6 for  $n$  values of 5, 6, and 7.

From these measurements it is apparent that the number of critical events in the retina required to produce a visual effect lies between 5 and 8. These values are in such good agreement with the results determined by the straightforward physical measurements already described that we must consider them as the actual number of quanta absorbed by the retina.

## X

*Physical Fluctuation and Biological Variation*

It is unimportant that the number of quanta delivered to the cornea is very much higher than the number finally involved in vision according to these measurements. This is because most of the light incident on the cornea is wasted and does not contribute to the initiation of a visual act. The amount falling on the cornea could be greatly increased by any arrangement in the eye which would act as a filter. Thus, the cornea and the lens might be pigmented, and this probably contributes to the fact that the oldest investigator (S.H.) actually requires the highest number of quanta incident on the cornea. Indeed, one might even put a filter immediately in front of the eye since the precise position of the filter in the optical system is immaterial. Nevertheless, the probability distributions would still remain the same, and by their shape would yield the magnitude of the number of events involved in the visual act.

It is necessary to amplify this point somewhat. Fluctuations are part of all physical systems, but they become significantly large only when the number of individual events, in the modern physical sense, is small. The general phenomenon is known as the shot-effect and has been studied extensively in electron emission, though it has wide application in the problem of measurements (Schottky, 1922; Barnes and Czerny, 1932). As a rough approximation, one may say that the range of variation is proportional to the square root of the number of individual events involved in the process.

In the optical system of our apparatus, the light from the ribbon filament lamp varies in intensity from moment to moment, but because the number of quanta emitted is enormous, the variation is almost too small to be measured. However, when the light intensity has been reduced first by the filters and wedge, then by the monochromators, then by the shutter, then by the ocular media, and finally by the retina itself, it has become so low that it represents only a few quanta per flash, and is therefore subject to great variation.

Barnes and Czerny (1932), and following them Brumberg and Vavilov (1933) realized that fluctuations must occur in the energy necessary for vision, and both groups of investigators looked for them. But they both missed the point of where the source of the fluctuations is and supposed it to be the energy deposited at the cornea. Brumberg and Vavilov even expected differences in the fluctuations for different wavelengths because of the greater energy required for seeing red light, for example, than blue-green light in conformity with the scotopic visibility curve of Fig. 5. However, the comparisons in Fig. 5 show that the differences in number of quanta required for vision in different parts of the spectrum record merely their relative absorption by visual purple. The number of absorbed quanta for an ultimate effect is the same regardless of wavelength and it is this number which sets the magnitude of the physical fluctuation encountered.

In deriving the curves of Fig. 6 for the quantitative statement of this physical fluctuation in terms of the Poisson probability distribution, we have made the single assumption that a constant number of quanta  $n$  must be absorbed by the retina in order for us to see a flash of light. Since it is conceivable, in view of the variability of an organism from moment to moment, that this value  $n$  is not constant, we have considered the consequences of assuming that the number  $n$  varies from time to time. The results show that biological variation is a factor of no great importance.

The situation may be best made clear by an example. Suppose that instead of  $n$  being constant, it varies between 4 and 8 quanta per visual act, and that the frequency with which 4, 5, 6, 7, and 8 quanta are necessary is distributed in terms of an ordinary probability distribution. The curves in Fig. 6 representing the frequency distributions for various values of  $n$  may then be weighted in this way and averaged. The average curve which is then secured is practically the same as the original Poisson distributions in Fig. 6, and may be fitted by the curves for  $n = 4$  or 5.

Thus, when biological variation is imposed upon the physical variation, there is no change in the essential characteristics of the physical distribution. Instead, the value of  $n$  merely falls below the average of the biological distribution, and is never below the lowest value in the distribution. This tells us that when, as in Fig. 7, the measurements yield  $n$  values of 5, 6, or 7, these numbers represent lower limiting values for the physical number of quanta. In other words, the only effect which biological variation has on the physical variation is to decrease the slope of the curves in Fig. 7 and thus make the apparent number of quanta smaller than the real number.

These considerations serve for understanding the meaning of the fluctuations shown by an organism in its response to a stimulus. It has generally been assumed that a constant stimulus, when presented frequently, remains constant, and that the fluctuations in response are an expression of the variations undergone by the organism. Indeed, this is one of the tenets of psychological measurements, and an elaborate structure of psychometrics has grown up on it as a basis (*cf.* Guilford, 1936).

The present evaluation of our measurements shows, however, that at the threshold the emphasis has been in the wrong place. At the threshold where only a few quanta of energy are involved, it is the stimulus which is variable, and the very nature of this physical variability determines the variation encountered between response and stimulus. Moreover, even when biological variation is introduced, it is the physical variation which essentially dominates the relationship.

This is at the absolute threshold. One may wonder, however, whether a differential threshold at any level of intensity may also involve a small number of events which determines the differentiation, and which may therefore be



subject to a similar physical variation as at the absolute threshold itself. Only experiment can decide this.

The fact that for the absolute visual threshold the number of quanta is small makes one realize the limitation set on vision by the quantum structure of light. Obviously the amount of energy required to stimulate any eye must be large enough to supply at least one quantum to the photosensitive material. No eye need be so sensitive as this. But it is a tribute to the excellence of natural selection that our own eye comes so remarkably close to the lowest limit.

#### SUMMARY

1. Direct measurements of the minimum energy required for threshold vision under optimal physiological conditions yield values between  $2.1$  and  $5.7 \times 10^{-10}$  ergs at the cornea, which correspond to between 54 and 148 quanta of blue-green light.

2. These values are at the cornea. To yield physiologically significant data they must be corrected for corneal reflection, which is 4 per cent; for ocular media absorption, which is almost precisely 50 per cent; and for retinal transmission, which is at least 80 per cent. Retinal transmission is derived from previous direct measurements and from new comparisons between the percentage absorption spectrum of visual purple with the dim-vision luminosity function. With these three corrections, the range of 54 to 148 quanta at the cornea becomes as an upper limit 5 to 14 quanta actually absorbed by the retinal rods.

3. This small number of quanta, in comparison with the large number of rods (500) involved, precludes any significant two quantum absorptions per rod, and means that in order to produce a visual effect, one quantum must be absorbed by each of 5 to 14 rods in the retina.

4. Because this number of individual events is so small, it may be derived from an independent statistical study of the relation between the intensity of a light flash and the frequency with which it is seen. Such experiments give values of 5 to 8 for the number of critical events involved at the threshold of vision. Biological variation does not alter these numbers essentially, and the agreement between the values measured directly and those derived from statistical considerations is therefore significant.

5. The results clarify the nature of the fluctuations shown by an organism in response to a stimulus. The general assumption has been that the stimulus is constant and the organism variable. The present considerations show, however, that at the threshold it is the stimulus which is variable, and that the properties of its variation determine the fluctuations found between response and stimulus.

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# THE EFFECT OF AUXINS ON PROTOPLASMIC STREAMING. III

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## I

### INTRODUCTION

Protoplasmic streaming in the *Avena* coleoptile is apparently closely related to a respiration process. This is particularly true for the acceleration of streaming which is produced by treatment with auxin. Experiments reported previously have shown that this acceleration is strictly dependent upon the accessibility of  $O_2$  (Sweeney, 1941) and have also given indirect evidence that the applied auxin increases the consumption of  $O_2$  by the tissues of the coleoptile (Thimann and Sweeney, 1937; Sweeney and Thimann, 1938). In the meantime the influence of auxin on  $O_2$  consumption has been reexamined (Commoner and Thimann, 1941). It was found that if the coleoptile sections were starved by soaking in water and then treated with malic or fumaric acid, the addition of auxin produces a marked rise in respiratory rate, and the increased respiration closely parallels the increase in growth.

The conclusion was drawn that auxin catalyzes a respiration system involving the 4-carbon acids, and that this respiration system, while accounting for only a small part of the total respiration of the coleoptile, controls a substantial part of the growth process.

Further, low concentrations of iodoacetic acid ( $5 \times 10^{-5}M$ ) completely inhibit the growth while lowering the total respiration only by about 10 per cent.

Thus the evidence indicates a close relationship between growth and respiration on the one hand, and between growth and the rate of protoplasmic streaming on the other. Since growth is apparently linked to a special part of the total oxidation, involving the 4-carbon acids, it is obviously of great interest to determine whether protoplasmic streaming is also related to this same oxidation reaction.

In order to continue making satisfactory measurements of the streaming rate, it was essential that the method of observation be made more objective and reliable. The present paper presents: first, a greatly improved method for the observation and recording of the protoplasmic streaming, and second, some experiments on the relation between the acceleration of streaming caused by auxin and the presence of malic acid.

## II

*Method*

Until recently, measurements of the rate of protoplasmic streaming have been made by timing the movement of particles between two points on an ocular micrometer by means of a stopwatch. This method requires considerable manipulation by the observer and introduces the opportunity for personal errors which are difficult to evaluate. For these reasons, a more mechanical and objective, as well as a more convenient method of measuring the streaming rate was desirable.

In all measurements of streaming, the basic assumption is that the particles move at the same rate as the protoplasm itself. The measurements will be more accurate the smaller the particle used in making them, since (a) the smaller the inertia of the particle the more nearly it approximates the rate of flow of the streaming, and (b) small particles meet fewer obstacles and thus their velocity is more constant. On this account the dark field method introduced by Olson and du Buy (1940) was not adopted. With dark field illumination the size of particles can only be determined by the relative intensity of the light they scatter. The brighter and more easily seen lines obtained by Olson and du Buy represent the motion of the larger particles and thus give the least accurate measurements.<sup>1</sup>

The principle of making observations by transmitted light as in our previous experiments was therefore decided upon. The rate of streaming, however, was measured by use of a comparison stream of variable but known velocity, in the following way:

A constant speed motor, driving a pulley, is mounted on a movable platform (see Fig. 1). This platform is moved back and forth on a screw by rotating a large wheel, *W*. As the motor advances toward the stationary pulley, the belt connecting motor and pulley is loosened and so the effective radius of the driving wheel on the motor is increased and the belt revolves faster. In this way the speed of the belt may be varied up to double its minimum rate.

The rate of rotation is reduced to a suitable value by introducing a set of reduction pulleys. The final small pulley drives a black elastic belt on which are sewn cut crystals. Snaps may be used in place of the crystal beads. These are illuminated by a dark room lamp, the light from which is filtered through water in a large flask, *C*. This same lamp serves as illumination for the binocular microscope (only stage, *S*, shown in diagram). The microscope stage, on which are mounted the slit oat coleoptiles being studied, is at the same level as the rotating elastic belt and bright reflecting crystal beads.

The crystals are superimposed on the cell in the field of the microscope by means of a camera lucida. To measure the rate of streaming, the bright spots from the beads

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<sup>1</sup> The infiltration procedure adopted by Olson and duBuy is open to a more serious objection which has been dealt with elsewhere (Sweeney, 1941).

are observed through the microscope and their speed is adjusted by means of the wheel until they move at the same rate as the fine particles in the cells.

To obtain the rate of streaming in  $\mu$  per second, the following calibration is necessary. The rate of rotation of the top section of the elastic belt is measured with a stopwatch at a series of different speeds. After a number of points have been taken, a calibration curve may be drawn showing the rate of rotation of the elastic belt, reduced to  $\mu$  per second, for all positions of the motor. Such a curve is shown in Fig. 2. Only the straight portion is used in making measurements of the rate of streaming. In this part of the curve, the average deviation from linearity is  $0.1\mu$ /second. Calibrations were repeated from time to time. While the slope of the calibration curve remained unchanged, the rate of rotation for any given position of the

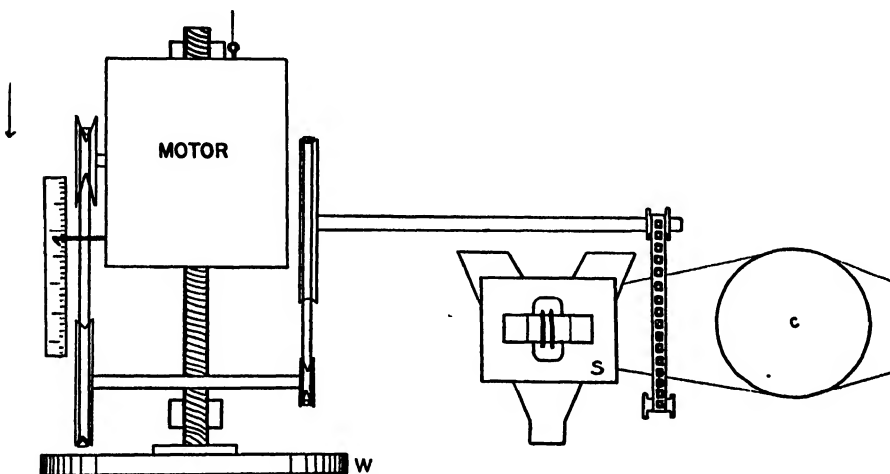


FIG. 1. Arrangement of apparatus (see text). The reference stream of crystals is to the right of the microscope stage *S*.

pointer on the scale increased progressively, the total shift during two years' use being  $0.75\mu$ /second.

The position of the motor is recorded by a wire passing over pulleys, attached to a block of brass, *B*, which travels up and down in a section of channel brass (Fig. 3). To this block is attached a heart lever (perpendicular to the plane of the diagram), magnifying the motion about two times, and writing with a fine pen on a slow kymograph. The kymograph used revolved at a reasonably constant speed of 8 mm./minute when kept tightly wound. In 30 minutes the record thus covered 240 mm. on millimeter paper with an average deviation of  $\pm 6.3$  mm. The general appearance of the whole apparatus is shown in the photograph (Fig. 4).

The acceleration of streaming produced by auxin may be temporary when studied in water solution, or of indefinite duration, when in presence of sugar (Sweeney and Thimann, 1938). In the former case the acceleration is given by the "total effect," *i.e.*, the area between the curve and the baseline which represents the initial rate. However, when the sugar is present, and the acceleration is maintained, the "total effect" is taken as the area under the curve during 30 minutes following the addition

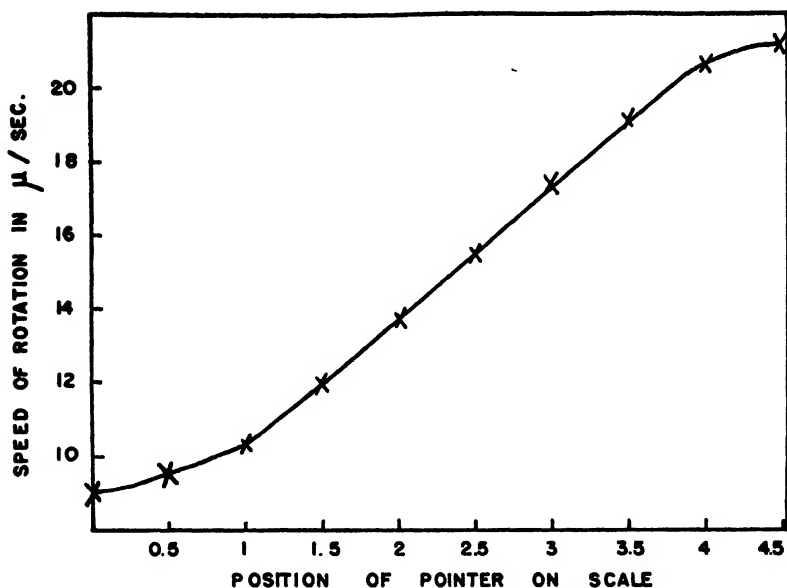
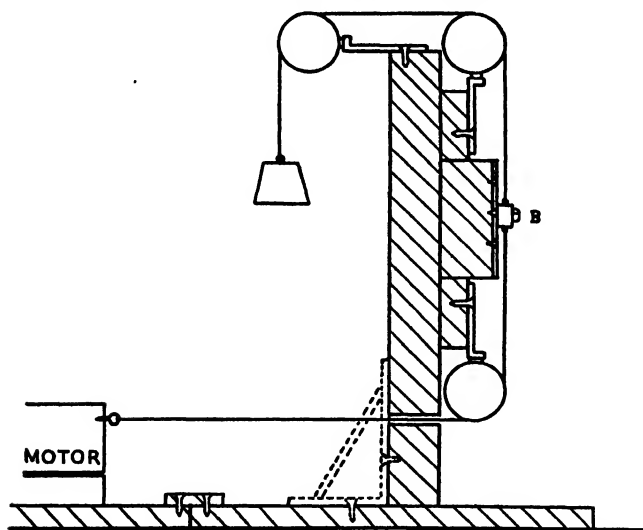


FIG. 2. Calibration curve for reference stream

FIG. 3. Device for recording position of the variable speed motor. The recording lever is attached to block B and is perpendicular to the plane of the paper (*cf.* Fig. 4).

of the solution bringing about acceleration. Since the acceleration of streaming with optimum concentrations of auxin is of the order of  $4\mu/\text{second}$  ( $= 40 \text{ mm. on the paper}$ ), the maximum error introduced by variation in the rate of rotation of the kymograph is then  $40 \times 6.3$  or  $250 \text{ mm.}^2$  As will be seen below, however, differences

in total effect up to about 1000 mm.<sup>2</sup> have to be neglected. A change in the rate of streaming of  $0.1\mu$ /second corresponds to 1 mm. on the paper.

Besides the small errors due to deviation from linearity in the calibration curve, and variations in speed of the kymograph, personal errors of observation and fluctuations in the protoplasm itself were assessed as follows: a coleoptile, mounted in pure H<sub>2</sub>O or sugar solution, was observed and the streaming rate recorded for several minutes. An assistant then changed the speed of the comparison stream by an

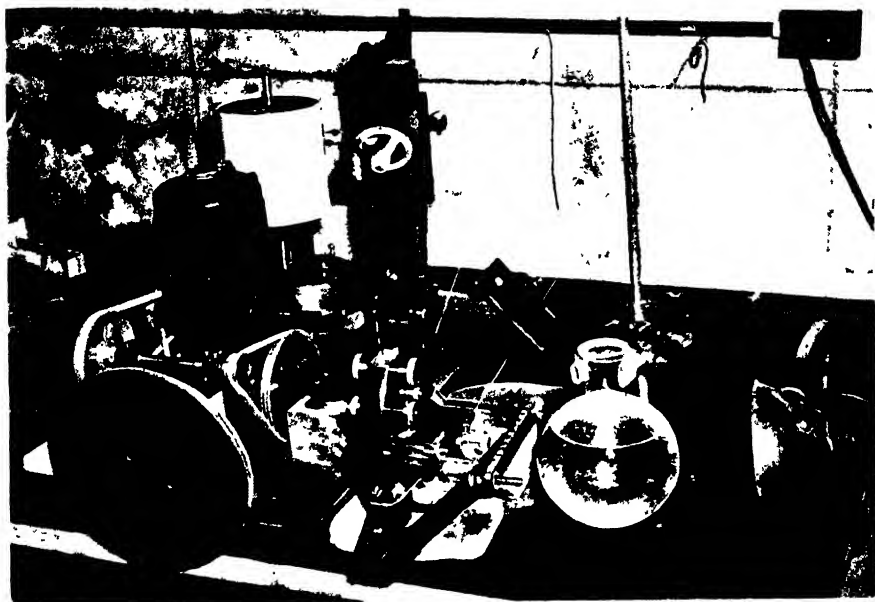


FIG. 4. For comparison with Figs. 1 and 3

unknown amount and the observer subsequently reset the rate to match that of the protoplasm. The results of two such tests were as follows:—

*Differences in the Rate of Streaming before and after Resetting*

	1/3/39		6/6/41
	$\mu$ /sec.		$\mu$ /sec.
	0.7		0.8
	0.55		0.1
	0.2		0.2
	0.3		0.2
	0.0		0.3
			0.0
			1.2
			0.5
Average difference .....	0.35		0.42
Average rate of streaming.....	13.5		14.3



The error of setting is thus of the order of  $\pm 0.4\mu/\text{second}$ . The optimum acceleration produced by auxin is about  $4\mu/\text{second}$  so that the error is about 10 per cent of the amount of the acceleration. In terms of the "total effect" this is equivalent, in a 30 minute record, to  $960 \text{ mm.}^2$  on the paper. Thus only total effects over  $1000 \text{ mm.}^2$  are significant. The effect of optimum auxin concentrations in sugar is of the order of  $4000 \text{ mm.}^2$ , and total effects as high as  $6000 \text{ mm.}^2$  have been obtained.

As before, the coleoptiles were sectioned lengthwise and mounted on a slide in water or sugar solution. The slide was examined under a total magnification of 575. Fresh solution was passed under the cover slip continuously from a drop at one side renewed every few minutes, and was drained off from the other side of the cover slip by means of a filter paper wick.

### III

#### *The Effect of Malate*

In the experiments on respiration it was found that malate had its optimum effect at 0.001 M: this concentration was therefore adopted in the present experiments. The auxin used was pure indole-3-acetic acid and the procedure was either to soak the coleoptile sections in malate before applying auxin, or to treat with a solution of auxin and malate. All auxin solutions were made up in 1 per cent fructose and oxygenated by bubbling  $\text{O}_2$  from a cylinder through the solution for about half an hour before use.

*Avena* coleoptiles, 4 days old from the time of soaking the seed, were used. The plants were grown under the usual conditions of the physiological dark room. Although coleoptiles 3 days old (the age of plants used in most growth experiments) were used in some of the early experiments because these young plants have a high rate of general respiration, and therefore the streaming is very oxygen-sensitive, plants 4 days old give more consistent streaming velocities and were used in the present and other more recent experiments.

Typical results of the influence of malate when added with sugar are shown in Fig. 5. The addition of malate without auxin had no effect on the rate of streaming under any of the conditions studied (Fig. 5, curve 1; Fig. 6, curve 4). In coleoptiles 4 days old the addition of malate to auxin solution of concentrations 0.1 and 1 mg./l. did not enhance the effect of auxin (Fig. 5, curves 2 and 3). These are the auxin concentrations which give optimum acceleration of streaming. Malate, however, increased the effect of small auxin concentrations. In coleoptiles 4 days old a concentration of indole-acetic acid of 0.005 mg./l. gave total effects just over  $1000 \text{ mm.}^2$ , *i.e.*, barely significant, while lower concentrations, 0.0025 and 0.001 mg./l., only rarely gave any effect at all. In presence of malate 0.001 M, however, auxin at 0.001 mg./l. gave very good acceleration of streaming (see Table I). The last experiment of Table I is shown in Fig. 5, curves 4 and 5.

The influence of treatments applied prior to the auxin was studied by cutting sections from coleoptiles 3 days old and soaking overnight in 1 per cent

fructose, 0.001 M malate, or water, or in fructose and malate. This was the procedure used by Commoner and Thimann (1941).

The sections used were 2 to 3 cm. long from which the tip (3 to 5 mm.) had been cut off. The following day these sections were slit lengthwise and treated with auxin 1 mg./l. or with auxin plus malate, records of the streaming rate being made as usual.

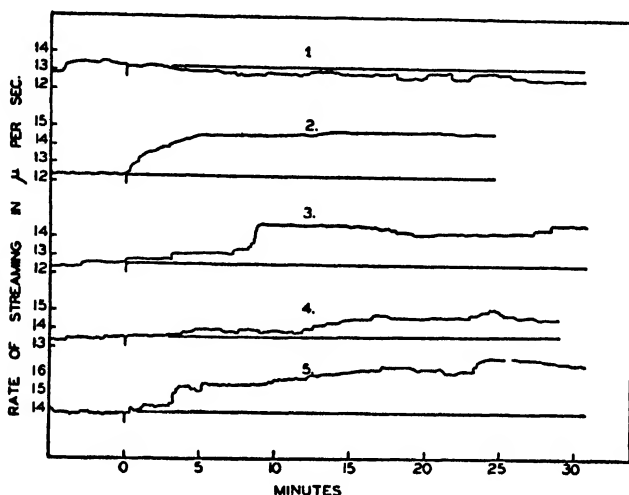


FIG. 5. The effect of malate on the velocity of streaming in coleoptiles 4 days old. Solutions to be tested were made up in oxygenated 1 per cent fructose, and were added to the coleoptile preparation at time zero, previous readings being made in 1 per cent fructose alone. In this and the following figures, all curves are typical of numerous replicate experiments.

Curve 1. Malate 0.001 M without auxin. Oct. 21, 1940.

Curve 2. Auxin 1 mg./l. Feb. 5, 1940.

Curve 3. Auxin 1 mg./l. plus malate 0.001 M. Feb. 5, 1940.

Curve 4. Auxin 0.001 mg./l. July 28, 1941.

Curve 5. Auxin 0.001 mg./l. plus malate 0.001 M. July 28, 1941.

When sections were soaked in 1 per cent fructose, or water only, auxin 1 mg./l. caused only a slight acceleration of streaming (see Table II). In other words, the period of soaking has the effect of very greatly reducing the sensitivity to auxin. It is as though some constituent of the coleoptile which is needed for the response has disappeared or has been used up. When these starved sections were treated with auxin and malate, however, a marked acceleration of streaming by auxin was obtained. The rate at the end of 30 minutes' treatment with auxin plus malate was equal to the rate reached in the best auxin concentrations with fresh 4-day plants, although the total effect for the first half-hour of treatment was smaller, since the rate increased

more slowly. When sections were soaked in malate either  $1\frac{1}{2}$  or 26 hours, then streaming accelerated promptly on addition of auxin and large total effects were obtained. In soaked coleoptiles, then, a large effect of malate in the presence of auxin may be observed. If malate is supplied, then soaked sections give as large an auxin reaction as do intact plants 4 days old.

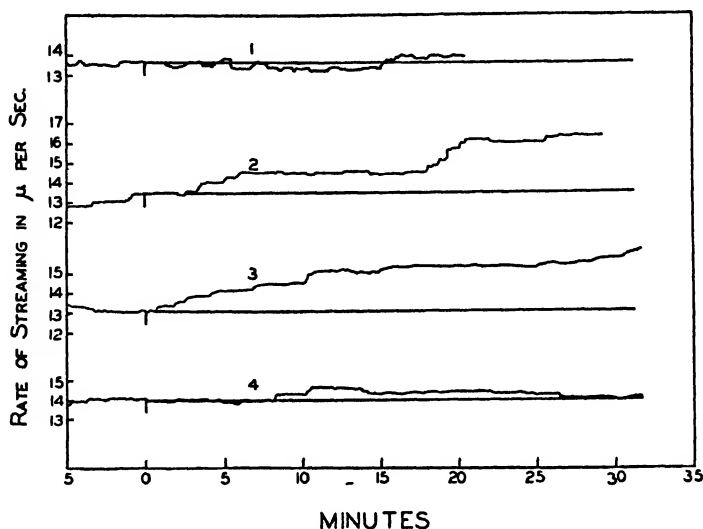


FIG. 6. The effect of malate on the rate of streaming in sections of coleoptiles cut when 3 days old and soaked overnight. Solutions to be tested were made up in oxygenated 1 per cent fructose (except curve 4) and added at time zero, previous readings of the rate of streaming being made in fructose alone.

Curve 1. Sections soaked in 1 per cent fructose 26 hours and treated with auxin 1 mg./l. Sept. 3, 1941.

Curve 2. Sections soaked in water 16½ hours and treated with auxin 1 mg./l. plus malate 0.001 M. Aug. 11, 1941.

Curve 3. Sections soaked in malate 0.001 M plus 1 per cent fructose 26 hours and treated with auxin 1 mg./l. Sept. 3, 1941.

Curve 4. Sections soaked in water 22 hours and treated with malate 0.001 M in water solution. Aug. 13, 1941.

Four curves, typical of those from which the data of Table II are derived, are shown in Fig. 6. They show: (curve 1) sections soaked in fructose do not respond to auxin alone, (curve 2) sections soaked in water do respond, though somewhat slowly, to auxin with malate, but not (curve 4) to malate alone: (curve 3) sections soaked in fructose with malate give an immediate large response to auxin alone.

Incidentally, the data above suggest that, while malate enters the cell and becomes effective more slowly than auxin, fairly large amounts have pene-

trated after 30 minutes. Soaking in malate before testing gave malate time to enter so that auxin might become effective at once. A soaking period of  $1\frac{1}{2}$  hours was as effective as one of 26 hours (Table II). A further experiment was designed to test the entrance of malate. A section soaked in sugar, *i.e.* one which would show only a small acceleration of streaming on the addi-

TABLE I

*Influence of Malate in Increasing the Sensitivity of 4 Day Old Plants to Low Concentrations of Indole-Acetic Acid (0.001 Mg./L.). Total Effects, Measured in  $mm.^2$*

Date	Without malate	With malate 0.001 M
	$mm.^2$	$mm.^2$
2/ 9/40	0	2595
2/21/40	0	2276*
5/15/40	0	{ 2600
		{ 2730
7/28/41	1137	4868

\* Soaked in malate 2 hours before experiment.

TABLE II

*Effect of Malate, 0.001M, on Streaming in Soaked Sections in the Presence of Auxin. Total Effects, Measured in  $mm.^2$*

Soaked in.....	Fructose 1 per cent		Water		Malate	
					26 hrs.	48 hrs.
Tested in.....	Auxin	Auxin and malate	Auxin	Auxin and malate	Auxin	Auxin
	$mm.^2$	$mm.^2$	$mm.^2$	$mm.^2$	$mm.^2$	$mm.^2$
	0	1962	1269	3384	4230	3453
	1160	2475	0	3900*		
	0	3085	1124	3147		
		2883				

\* Section soaked in malate  $1\frac{1}{2}$  hours before testing.

tion of auxin without malate, was treated with auxin and malate for 37 minutes. During this time the streaming velocity increased from 13.9 to 16.2  $\mu$ /second. The malate was then rinsed off the section thoroughly and auxin solution without malate was substituted. If no malate had entered the cell, then the streaming rate should have fallen to the control level at once. However, streaming velocity remained at 16.2  $\mu$ /second for at least 30 minutes after malate was removed from the external solution.

As the coleoptile grows older, the auxin effect on streaming disappears. The age at which cells no longer respond to auxin (0.1 to 1 mg./l.) varies be-

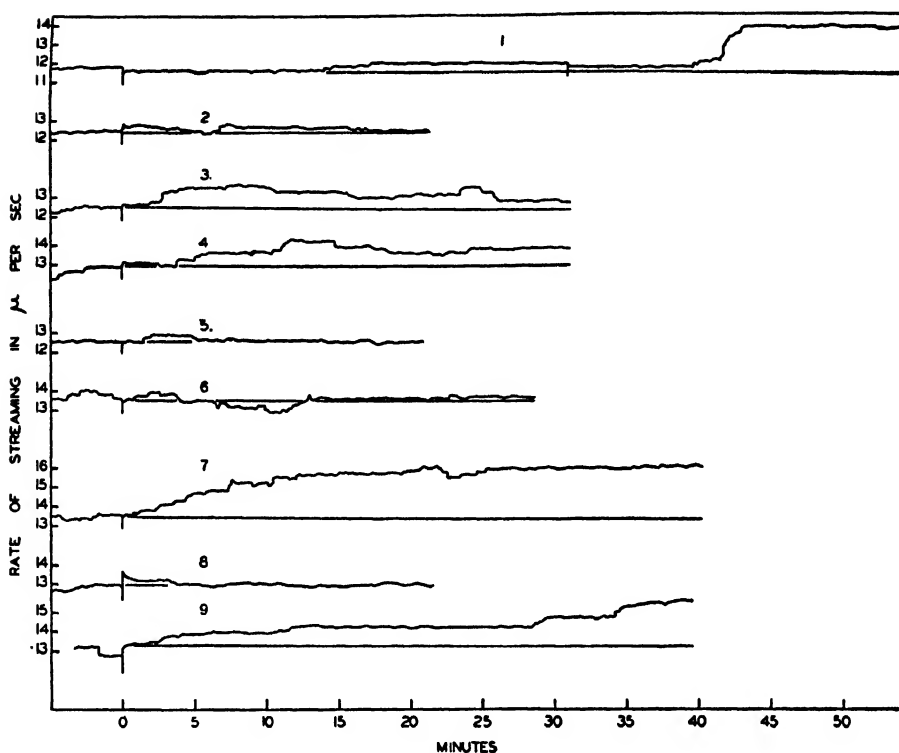


FIG. 7.

Curve 1. The effect of auxin, and auxin and malate on streaming in the *Avena* coleoptile 6 days old. Sections treated at time zero with auxin 0.1 mg./l. in oxygenated 1 per cent fructose solution, previous readings being made in fructose alone. At 32 minutes, the auxin solution was replaced by auxin 0.1 mg./l. with malate 0.001 M in fructose. Feb. 16, 1940.

Curves 2-9. The effect of different concentrations of iodoacetate on the rate of streaming in coleoptiles 4 days old. Solutions made up in oxygenated 1 per cent fructose and added at time zero, previous readings being made in fructose alone.

Curve 2. Iodoacetate  $1 \times 10^{-7}$  M. Apr. 9, 1940.

Curve 3. Iodoacetate  $5 \times 10^{-7}$  M. Mar. 6, 1940.

Curve 4. Iodoacetate  $1 \times 10^{-6}$  M. Feb. 28, 1940.

Curve 5. Iodoacetate  $5 \times 10^{-6}$  M. July 31, 1940.

Curve 6. Iodoacetate  $2 \times 10^{-4}$  M. Oct. 31, 1941.

Curve 7. Indole-3-acetic acid 1 mg./l. Aug. 15, 1941.

Curve 8. Indole-3-acetic acid 1 mg./l. plus iodoacetate  $5 \times 10^{-6}$  M. Mar. 1, 1940.

Curve 9. Indole-3-acetic acid 1 mg./l. plus iodoacetate  $5 \times 10^{-6}$  M plus malate 0.001 M. Mar. 1, 1940.

tween 6 and 8 days from time of soaking of the seed. When the protoplasm will no longer react to auxin in sugar solution, acceleration of streaming may be obtained by adding malate to the auxin solution (Fig. 7, curve 1). However, sections of plants 4 days old cut and placed on combs floating on auxin solution 1 mg./l. did not grow more than a few per cent. Streaming in the same material gives its maximum response to auxin of this concentration. This would suggest that the ability of auxin to cause growth is lost while the ability of the protoplasm to respond well to auxin is retained.

#### IV

##### *The Effect of Iodoacetate*

It was shown by Commoner and Thimann that iodoacetate at  $5 \times 10^{-5}$  M completely inhibits growth without affecting more than a very small part of the total respiration, and it was deduced that at this concentration it inhibits only the "growth" respiration. At higher concentrations larger inhibitions of respiration were found. The influence of low concentrations of iodoacetate on streaming were therefore studied.

With very small concentrations of iodoacetate without auxin, ( $5 \times 10^{-7}$  M,  $1 \times 10^{-6}$  M; Fig. 7, curves 3 and 4), small accelerations of streaming were observed (*cf.* the acceleration of growth found for these concentrations by Commoner and Thimann). Larger concentrations, as high as  $2 \times 10^{-4}$  M had no effect alone on streaming within 30 minutes (Fig. 7, curves 5 and 6). However, concentrations of  $5 \times 10^{-5}$  M potassium iodoacetate completely inhibited the acceleration of streaming by auxin 1 mg./l., when coleoptiles were treated with mixtures of auxin and iodoacetate (Fig. 7, curves 7 and 8). Malate 0.001 M partly overcame the inhibitory effect of iodoacetate (Fig. 7, curve 9). Iodoacetate  $1 \times 10^{-6}$  M did not affect the acceleration of streaming by auxin. The correspondence with the concentrations of iodoacetate effective on respiration is thus very close.

#### V

##### DISCUSSION

The parallel between the effects of malate on growth and respiration and on the acceleration of streaming is striking. Malate alone is without effect on respiration and this is true also of the rate of streaming. Whether or not malate influences the effect of auxin on streaming depends on the condition of the coleoptile tissue. Under optimal conditions for the effect of auxin alone (fresh sections of 4-day plants, oxygenated solution containing optimum auxin concentration), malate exerts no effect. Evidently here the upper limit of possible acceleration of streaming has been reached. Under the same conditions except with auxin concentration at the lowest limit of effect, malate

greatly increases the sensitivity of the cells to auxin. This suggests that here, as with sugar (Schneider, 1938), the auxin reaction is dependent on both auxin and malate concentration at the same time, and therefore the effect of these substances does not follow the classical limiting factor scheme of Blackman.

In old plants, or in sections soaked for many hours in fructose or in water alone, the response to auxin practically disappears, and the addition of malate brings it back to normal or maximal. The obvious deduction is that these are the conditions under which malate is depleted from the cells.

Streaming in soaked sections behaves as predicted from the respiration measurements, that is, as if the acceleration on addition of auxin were brought about by acceleration of the 4-carbon acid respiration. In sections soaked in water or sugar, auxin accelerates streaming only in the presence of malate and this is an exact parallel of the results of respiration studies with similar cells. It should be noted that when sections are cut at 3 days and soaked overnight they are no longer comparable with fresh sections from plants 4 days old. This point must be borne in mind in making quantitative correlations of streaming data with measurements of growth of sections.

The parallel between growth and streaming may be extended to include the experiments on iodoacetate, which has an opposite effect to that of malate. Potassium iodoacetate,  $5 \times 10^{-6}$  M and above, inhibits the effect of auxin on growth, and the same concentrations also inhibit the acceleration of streaming by auxin. Lower concentrations of iodoacetate ( $1 \times 10^{-6}$  M) bring about small accelerations of both streaming and respiration. It is important, however, that the effect of iodoacetate is exerted on the *acceleration* produced by auxin and not on the basal rate of streaming. The acceleration could be completely inhibited while the basal rate remained unaffected even by concentrations 10 times as great. Hence this basal rate of streaming cannot be in large part controlled by auxin. This is substantiated by the observation that control rates do not vary with soaking nor with the age of the coleoptile, although auxin production, at least, falls off with age. The streaming brought about by auxin may be clearly separated from that before treatment with auxin. In fact no auxin-controlled streaming has been demonstrated in the untreated cell, although some auxin is undoubtedly present in these cells. It is well to make clear, then, that the basal streaming rate is dependent on some quite other system than that controlled by auxin.

The experiments on coleoptiles of different ages present evidence that cessation of growth may take place while the cells are still able to respond internally to auxin. Presumably growth is prevented by rigidity of the cell walls. Kerr and Thimann (unpublished) have shown that secondary wall begins to form in the basal cells of the coleoptile late on the 3rd day. The micelles in the secondary wall are oriented almost longitudinally and thus would powerfully prevent elongation. This has also been brought out by experiments on cells

kept at low temperature where deposition of wall is prevented and growth continues longer than at room temperature (Heyn, 1931; Heyn and van Overbeek, 1931; Bonner, 1934). Ageing may thus be divided into at least two processes, the first a thickening of the cell wall, the second the exhaustion of malate reserves. The second process may be reversed by addition of malate.

It may be concluded in general that the presence of malate is a necessary condition for the effect of auxin both on streaming and on growth, and that iodoacetate in the proper concentration acts as a clear cut inhibitor for both processes.

## VI

### SUMMARY

1. A new method is described which gives a continuous record of the absolute rate of protoplasmic streaming in epidermal cells of the *Avena* coleoptile.

2. With this method a study was made of the influence of malate and iodoacetate on streaming velocity, in order to make correlations with the previously established effects of these substances on growth and respiration.

3. In the presence of optimum concentrations of indole-3-acetic acid in freshly cut sections, malate had no effect on streaming. In the presence of very low concentrations of the auxin, however, malate increased the range of response, so that the threshold of auxin sensitivity was lowered some ten times by the malate. Malate alone had no effect on streaming.

4. In coleoptile sections, soaked overnight in sugar solution or in water, the acceleration of streaming normally caused by auxin almost disappears, but the presence of malate causes large accelerations of streaming by the auxin.

5. Similarly, in sections from old coleoptiles, which no longer show acceleration of streaming by auxin, the acceleration is restored when malate is added together with the auxin.

6. Malate does not enter the cell as rapidly as does auxin, but easily detectable amounts penetrate within 30 minutes.

7. Iodoacetate in the concentration which inhibits growth ( $5 \times 10^{-5}$  M) completely inhibits the acceleration of streaming by auxin. In still lower concentrations iodoacetate slightly accelerates streaming. Higher concentrations, up to  $2 \times 10^{-4}$  M, did not reduce the rate of streaming below that of controls without auxin. The effect of iodoacetate is therefore to inhibit the acceleration caused by auxin and not to affect the basal streaming rate.

8. It is concluded that, just as for growth and respiration, malate is necessary for the response to auxin shown by acceleration of streaming. This further strengthens the triple parallel between the effects of auxin on streaming, growth, and respiration, all of which are apparently mediated by the 4-carbon acid system.



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# HYDROSTATIC PRESSURE AND TEMPERATURE IN RELATION TO STIMULATION AND CYCLOSIS IN *NITELLA FLEXILIS*

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This study was undertaken for two reasons, (1) to determine if *Nitella* can be stimulated by a sudden increase or decrease of hydrostatic pressure; (2) to compare the cyclosis velocity under pressure in *Nitella* with that in *Elodea*, which Marsland (1939) found to be progressively slowed as the pressure was increased. This slowing was accompanied by a similar progressive decrease in viscosity, as measured by the rate of sedimentation of chloroplasts within the *Elodea* cell under the influence of centrifugal force.

As is well known, response of *Nitella* cells is observed after stimulation by touching or bending (see Ewart, 1903; also Osterhout and Hill, 1931), application of solutions such as KCl or chloroform (Osterhout and Hill, 1930), cold or heat (Dutrochet, 1838), electrically (Dutrochet, 1838; Hörmann, 1898) or by flashes of ultraviolet light (Harvey, 1942). The criterion of response may be an action potential or a stopping of the cyclosis, which has been called "shock stoppage."

*Nitella* does not under all circumstances obey the all-or-none law, since the magnitude of the action potential may vary and conduction with a decrement may occur (Blinks, Harris and Osterhout, 1929). Hill (1941) studied the relation between stimulation and cyclosis in *Nitella* and concluded that electrical stimulation is accompanied by an action potential only if the stimulus is of sufficient intensity. A weak stimulating voltage will cause the cyclosis gradually to slow down and come to a stop, no action potential appearing, while a higher voltage causes an abrupt stop with an action potential.

In addition to the normal action potential, which is propagated at a rate of 2 to 3 cm. per second, electrical variations may occur in *Nitella* due to mechanical disturbances which travel along the cell with high velocity and stimulate each region as they pass (Osterhout and Hill, 1931), as well as death waves, also travelling with high velocity (Osterhout and Harris, 1928, 1929). The response may thus be decidedly complicated but shock stoppage of cyclosis is a sure sign of stimulation.

Since the pressure chamber used in these experiments contained no electrodes for leading off the action potential, the criterion of stimulation by pressure was a sudden stopping of cyclosis. In addition we may expect pressure to affect cyclosis directly as in the *Elodea* experiments of Marsland (1939).

As *Nitella* is particularly sensitive to mechanical stimulation, the first experiments were directed to determining if a pressure wave in water at atmospheric pressure would cause shock stoppage of cyclosis. For this purpose a brass chamber (10 cm. high and 5.1 cm. internal diameter) was made, which contained a glass window 6.2 mm. thick in the lower end and in the other a solid brass piston containing a small hole fitted with a lucite plug. The chamber was completely filled with pond water (excluding air bubbles) and a *Nitella* cell, which rested on the glass bottom and could be observed with an inverted microscope by means of a light beam that passed through the small hole fitted with lucite in the piston head. The piston head rested on the water column and could be given a sharp blow with a hammer or with a steel ball falling from a given height. A pressure wave could thus be started, which travelled downward through the water to strike the *Nitella* cell, whose cyclosis was continually observed. It was found that such pressure waves resulting from blows with a hammer or from a steel ball of 534 gm. weight falling 145 cm. (energy = 7.6 joules) did not affect cyclosis, unless a glancing blow, that actually moved the *Nitella* cell, struck the cylinder. A pressure wave like the above in water at atmospheric pressure may therefore be eliminated as a stimulus to stoppage of cyclosis.<sup>1</sup>

The next experiments were with sudden changes of hydrostatic pressure. These were applied to the *Nitella* cell in a special pressure chamber with glass windows, similar to that used by Marsland (1939), so that the *Nitella* could be observed with an inverted microscope during the increase in pressure. The pump was a 7 ton hydraulic auto truck jack converted to deliver hydrostatic pressures up to 10,000 lbs. per square inch.<sup>2</sup>

The results depended on the *Nitella* cell. In general sudden increases in pressure from 0 to 5000 lbs. per square inch or 5000 to 9000 lbs. or increases in steps of 1000 lbs. each do not stop cyclosis. Some cells, however, will occasionally stop on a sudden increase of pressure and others whose cyclosis does not stop on changes in pressure up to 5000 lbs. will stop on changing from 5000 to 6000 or 6000 to 7000 lbs. or 7000 to 8000 lbs. These are exceptions but nevertheless the stoppage of cyclosis can only be attributed to stimulation by pressure and not to joggling of the pressure chamber or movement of the *Nitella* cell in the chamber. The response to sudden pressure increase is somewhat like the response to sudden ultraviolet light stimulation, as described by Harvey (1942), where an action potential appeared in only about one-third of the cells exposed to the ultraviolet light flash.

When the pressure on *Nitella* was suddenly released from varying high pressures (9000 to 5000 lbs.) to atmospheric pressure, the cyclosis stopped in 50 per cent of twenty-two trials. The behavior depended on the *Nitella* cell used in the study. Stopping of cyclosis did not necessarily occur as a result of a slight movement in the

<sup>1</sup> Ewart (1903, p. 72) quotes Hörmann (1898) as finding that rapid changes in pressure up to 2 atmospheres do not cause shock stoppage of cyclosis in *Nitella syn-carpa*, but that a smart blow on a piston head connected with the chamber containing *Nitella* will cause temporary cessation of streaming. The conditions of the experiment are evidently important.

<sup>2</sup> One atmosphere = 14.7 lbs. per square inch = 1033 gm. per sq. cm. 1000 lbs. per square inch = 68 atmospheres = 70.2 kg. per square centimeter.

field and could again be attributed only to stimulation. Perhaps the greater certainty of stimulation on release of pressure is due to the fact that release of pressure can always be attained more suddenly by turning a valve, whereas increase of pressure must be built up by pumping the handle of the hydraulic jack.

In order to complete the picture of pressure effects, experiments were carried out to see if a sudden decrease of the atmospheric pressure over water containing the *Nitella* cells would cause shock stoppage of cyclosis. The cells were in a glass chamber with a plane glass bottom so that observation could be made with an inverted microscope. The chamber could be connected by a two-way stop-cock and pressure tubing to a large evacuated desiccator or to the air. By turning the cock, sudden decreases or increases of pressure (20 mm. to 760 mm. Hg) could be applied to the *Nitella* cells but shock stoppage was never observed to occur.

The direct effect of pressure on rate of cyclosis apart from stimulation is indicated in Fig. 1, in which the cyclosis rate in micra per second is plotted against the pressure in pounds per square inch, as the pressure is raised in steps of 1000 lbs. per square inch. Cyclosis rate was measured at 23°C. by timing the movement of the granules in the protoplasm across the lines of a square ruled micrometer in the eye-piece. Since the individual granules vary considerably in rate the figures are averages of 5 or more readings. 3 to 5 minutes elapsed between the 1000 lb. changes in pressure. Table I gives the data for all successful experiments.

The slowing of cyclosis by pressure in *Nitella* is not as regular as in *Elodea*, and different cells behave differently. Some show a progressive slowing of cyclosis as the pressure is increased in increments of 1000 lbs. per square inch while in others there is little change in rate until 4000 lbs. is reached and then a progressive decrease. Curve A is the average of all readings on three different cells taken on different days, in which there was a progressive slowing of cyclosis rate as the pressure was increased. Curve B is also the average of all readings on three different cells taken on different days where the slowing of cyclosis began at 4000 lbs. At 9000 lbs. the rate is only about  $\frac{1}{2}$  of that at atmospheric pressure and cyclosis does not stop completely at 10,000 lbs. per square inch. Marsland (1939) found cyclosis to stop in *Elodea* between 6000 and 7500 lbs.

If the pressure is suddenly released and no stimulation occurs, the rate generally returns to very nearly the original rate at atmospheric pressure. The same is true when the pressure is decreased in steps of 1000 lbs. per square inch. The return of cyclosis velocity is over approximately the same course as when the pressure is increased. Some cells, however, which have been kept at the high pressure for some time (30 minutes) appear to be injured by the pressure treatment and the cyclosis is permanently slowed.

*Nitella* cells thus behave in most respects in the same way that *Elodea* cells do, except that the cyclosis rate is complicated by phenomena connected with stimulation. From analogy with other experiments on pressure, which brings

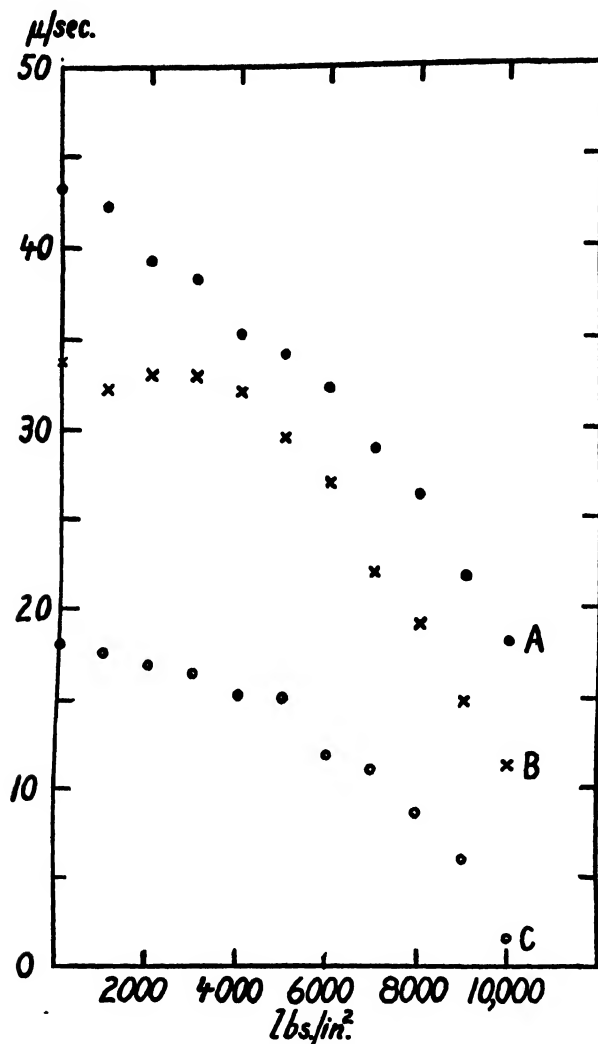


FIG. 1. Cyclosis rate in micra per second as a function of hydrostatic pressure. A, average of three experiments on three different cells at 23°C. where the pressure effects are marked from the start. B, average of three experiments on three different cells at 23°C., where the pressure effects begin after 4000 lbs. per square inch. C, experiment with a single cell at 10°C. Cyclosis returned to 18  $\mu$  per second at 10°C., when the pressure was removed and to the original value (41  $\mu$  per second) at 24°C. when the temperature was raised.

about solation in many cells (see Marsland, 1942), the decreased viscosity of the protoplasm may be the cause of the slowing of cyclosis but a similar argu-

ment applied to the effects of temperature on cyclosis would lead to another conclusion, namely, that decreasing viscosity with increasing temperature should also result in less rapid cyclosis. Actually cyclosis rate is greatly increased by rise of temperature. Moreover, if viscosity changes determine the rate of cyclosis, one might argue that cyclosis which has been slowed (be-

TABLE I  
Summary of *Nitella* Pressure Experiments at 23°C

Date	lbs./in <sup>2</sup> ...0	1000	2000	3000	4000	5000	6000	7000	8000	9000	10,000
1942	$\mu$ per sec.	$\mu$ per sec.	$\mu$ per sec.	$\mu$ per sec.	$\mu$ per sec.	$\mu$ per sec.	$\mu$ per sec.	$\mu$ per sec.	$\mu$ per sec.	$\mu$ per sec.	$\mu$ per sec.
Apr. 4 .....	45.6	44.0	38.8	41.0	38.5	38.8	35.1	31.5	29.3	23.1	19.3
Apr. 3 .....	51.0	52.0	49.0	45.4	41.0	39.1	37.5	29.4	26.0	20.8	14.7
Mar. 28 (1) .....	33.4	30.8	30.8	28.2	25.8	25.3	24.7	26.0	23.5	(21.5)	(20.0)
Mar. 28 (2)* .....	36.7	35.4	33.7	26.3	28.4	26.7	23.4				
Feb. 20 .....	58.3	54.5	56.0	52.5	48.8	48.2	42.4				
Feb. 24 (1) .....	49.1	47.0	44.4	42.4	38.0	37.8	(35.0)				
Average .....	45.7	43.9	41.9	39.3	36.7	35.9	33.0				
Mar. 24 (1) .....	27.7	26.3	27.1	26.7	26.2	25.7	25.0	21.8	18.6	13.8	12.4
Mar. 24 (2)* .....	24.3	22.7	22.7	22.4	21.1	18.3	16.7	16.8			
Mar. 26 (1) .....	27.0	26.0	25.7	24.1	24.7	22.6	(20.0)				
Mar. 20 .....	42.5	38.5	40.8	37.8	38.2	32.8	29.2	24.6	22.2	19.0	(15.0)
Feb. 24 (2) .....	49.8	45.7	47.2	42.3	28.9	24.6	(20.0)				
Feb. 5 (1) .....	31.4	31.4	31.4	35.0	32.4	30.0	27.5	22.0	16.9	11.6	(07.0)
Feb. 5 (2)* .....	36.6	34.0	31.4	31.4	31.4	24.4	23.6				
Average .....	34.1	32.0	32.3	31.3	28.9	25.4	23.1				
Grand average .....	39.9	37.9	37.1	35.3	32.8	30.6	28.0				
Apr. 4 (dec.) .....	46.5	47.7	44.5	41.7	43.5	41.3	34.5	29.0	28.1	18.8	19.3

The figures give the cyclosis velocity at increasing pressures. Those in parentheses are extrapolations from a graph. An asterisk indicates a second experiment with the same cell. *Nitella* cells in the first six experiments showed continuously decreasing cyclosis velocity with increasing pressure. Cells in the last seven experiments were little affected until after 3000 lbs. per square inch pressure. The last line gives values for cyclosis in the Apr. 4 experiment as pressures are decreased from 10,000 lbs. per square inch.

cause of viscous protoplasm) at a low temperature could be increased by raising the pressure and hence decreasing the viscosity, but such is not the case, as I have determined by experiment (see below).

However, according to Marsland's (1942) theory, *Elodea* cyclosis is due to a wave of sol  $\rightleftharpoons$  gel transformation, accompanied by change in volume, which passes around the cell. The volume changes are responsible for the circulatory movement. Pressure slows the cyclosis by preventing this sol  $\rightleftharpoons$  gel

change, by forcing the equilibrium toward the sol state making the protoplasm mere liquid. Pressure thus acts not by continuously decreasing the viscosity in the ordinary sense but by preventing the sol  $\rightarrow$  gel transformation.

The whole question of pressure effects is intimately connected with temperature. Recently Johnson, Brown, and Marsland (1942) have described an important pressure-temperature relationship in luminous bacteria. These forms have a well defined temperature optimum for maximum luminescence intensity, ranging from 20–32°C., depending primarily on the bacterial species. Above this temperature the light intensity decreases reversibly, so that lowering the temperature will restore the original luminescence if the bacteria are not kept too long at the higher temperature. An increase of pressure will also increase the luminescence intensity at temperatures above the optimum. This striking result is explained by the effect of pressure in reversing the equilibrium between active and reversibly heat-denatured luciferase, the enzyme responsible for luminescent oxidations in organisms. Rise in temperature shifts the equilibrium toward the denatured enzyme, while rise in pressure shifts the equilibrium in the opposite direction. At temperatures above the optimum the lowered light intensity is due to lowered active luciferase concentration and pressure restores the luminescence by shifting the equilibrium toward greater concentration of active luciferase. It would be interesting to know if a similar pressure-temperature relationship exists for cyclosis.

The effect of temperature on cyclosis in *Characeae* is quite well known. It has been studied by Velten (1876), using *Chara foetida*, who found a maximum rate at 34°C. and by Nägeli (1860), using *Nitella syncarpa*, where the maximum is at 37°C., as well as by Lambers (1925, 1926) and Romijn (1931). Lambers (1925) gives a curve for cyclosis rate and temperature in *Nitella mucronata* which is very nearly a straight line between 2°C. (8 $\mu$  per second) and 34°C. (100 $\mu$  per second). Above 34°C. the cyclosis rate depends on the time of heating.

The *Nitella flexilis* used in these pressure experiments had an optimum temperature of cyclosis of about 35–36°C., when heated at a rate of 10° in 30 minutes, with rapid falling off of rate with higher temperatures. At 38.5° the rate was the same as that at 22°C. If kept at the high temperatures there was a progressive falling off in rate which made the experiments difficult to carry out. Therefore the *Nitella* cells within the pressure chamber at one atmosphere pressure were quickly brought to various temperatures ranging from 32°–39°C. At 32°C. the cyclosis rate was greater than at 22°C., whereas at 39°C., the rate was less than at 22°C. When the proper high temperature was attained, the pressure was raised to 3000 lbs. per square inch, where the cyclosis rate was quickly measured, and then to 5000 to 6000 lbs. per square inch where the cyclosis rate was again quickly measured.

In no case did cyclosis rate increase as a result of the increase in pressure.

There was always a decrease in rate, slight at 3000 lbs. per square inch and great at 6000 lbs. per square inch. If raised only to 32°C. there was a return of cyclosis to the normal value at 32° when the pressure was released but when raised to higher temperatures (37° or 38°) the cyclosis was permanently slowed when the pressure was released and did not completely recover at room temperatures except after a long time.

Because of the rapid decline in cyclosis rate at temperatures above the optimum and the time factor, it is not feasible to work out curves showing the effect of pressure on cyclosis at various critical temperatures. The material is not good for pressure-temperature studies, because the optimum temperature is so near the maximum temperature at which cyclosis can continue. In luminous bacteria there is a rather long temperature step between the optimum and the maximum temperature at which these organisms can luminesce. However, I have convinced myself that increase in pressure never increases the cyclosis rate of *Nitella* at temperatures near or above the optimum as is the case for the luminescence intensity of luminous bacteria. Probably the pressure effects on cyclosis, ameoboid movement, cell division, and pigment flow in chromatophores are in another category from pressure effects on the luminescent reaction, which primarily depends on catalytic oxidation. Luminescence of bacteria is more comparable to such activities as muscle contraction, nerve conduction, and ciliary or flagellar movement, where moderate pressures may increase the activity.

To complete the picture of temperature effects, 10°C. was selected as a convenient point for studying the effect of pressure at temperatures well below the optimum and well below the temperature of the room in which the plants had been growing all winter. At 10° the rate is about half that at 20°. However, when the pressure is increased at this low temperature, the rate of cyclosis is always decreased, relatively little for the first 5000 lbs. per square inch but markedly at 6000 to 10000 lbs. per square inch. When the pressure was removed, the rate returned to that which is normal at 10°C. and when the temperature was raised to 20°C. the rate returned to that characteristic of the higher temperature. A curve is given in Fig. 1, C. Therefore, if the slowing of cyclosis at low temperatures is due to increased viscosity of the protoplasm, as previously explained, a solution by increased pressure at this low temperature cannot counteract the slowing. However, we do not know how temperature (or pressure) affects the motive force for cyclosis (whatever it may be) apart from an effect on viscosity, and such knowledge is essential for extended discussion.

There is another reason why temperature studies on cyclosis are difficult. I can confirm the older observations of Hofmeister (1867), Hörmann (1898), Lambers (1925), Umrath (1930), Romijn (1931), and Hill (1935) that a sudden decrease in temperature will stimulate, causing shock stoppage of cyclosis, and in addition have confirmed Dutrochet (1838), that sudden increases in temperature will also cause shock stoppage. Time must always be allowed for recovery from this shock effect.



I take pleasure in acknowledging the aid of my research assistant, Mr. Harold Towne, who made many of the observations on the rate of cyclosis at different pressures.

#### SUMMARY

*Nitella flexilis* cells are not stimulated to "shock stoppage" of cyclosis by suddenly evacuating the air over the water or on sudden readmission of air, or on suddenly striking a piston in the water-filled chamber in which they are kept with a ball whose energy is 7.6 joules, provided the *Nitella* cell is not moved by currents against the side of the chamber.

Sudden increases in hydrostatic pressure from zero to 1000 lbs. or 0 to 5000 lbs. per square inch or 5000 to 9000 lbs. per square inch usually do not stimulate to "shock stoppage" of cyclosis, but some cells are stimulated. Sudden decreases of pressure are more likely to stimulate, again with variation depending on the cell.

In the absence of stimulation, the cyclosis velocity at 23°C. slows as the pressure is increased in steps of 1000 lbs. per square inch. In some cells a regular slowing is observed, in others there is little slowing until 4000 to 6000 lbs. per square inch, when a rapid slowing appears, with only 50 per cent to 30 per cent of the original velocity at 9000 lbs. per square inch. The cyclosis does not completely stop at 10000 lbs. per square inch. The pressure effect is reversible unless the cells have been kept too long at the high pressure.

At low temperatures (10°C.) and at temperatures near and above (32°–38°C.) the optimum temperature for maximum cyclosis (35–36°C.) pressures of 3000 to 6000 lbs. per square inch cause only further slowing of cyclosis, with no reversal of the temperature effect, such as has been observed in pressure-temperature studies on the luminescence of luminous bacteria.

Sudden increase in temperature may cause shock stoppage of cyclosis as well as sudden decrease in temperature.

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# ACCUMULATION OF SALT AND PERMEABILITY IN PLANT CELLS

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This paper represents a further report on the progress of investigations originally described by Hoagland and Broyer<sup>1</sup> and by Prevot and Steward.<sup>2</sup> (Compare also investigations of Steward and associates on storage tissues.<sup>3</sup>) These investigations have as their special objective the development of knowledge of the relation of cell metabolism to the accumulation of salt against concentration or activity gradients by root cells. The general technique of experimentation on roots has already been described.<sup>1</sup> As one feature of this technique the internal concentrations of salt were usually determined on sap expressed from the root tissues after killing them by rapid freezing. This procedure is capable of yielding consistent and useful results on the influence of many factors of the internal and external environment on the absorption and accumulation of salt.<sup>4</sup> We are of the opinion that most of the salt recovered in the expressed sap is located in cell vacuoles, large or small. Obviously, however, the sap derived from complex tissues does not represent so definite a component of living cells as does the vacuolar sap that can be removed from large coenocytic algal cells. It is of interest, therefore, to consider another method of studying the movement of salt against gradients in root systems. Investigation of the so-called bleeding sap obtained from the roots of barley or other plants when the shoot is abscised a short distance above the root system provides another method of study. There are various reasons for believing that this sap emerges primarily from xylem vessels.

## *Comparison of Composition of Exudates and of Expressed Sap from Roots*

We have made a number of comparisons of the composition of sap expressed from barley root tissues with that of the exudation fluid, in experiments conducted on plants absorbing salt from dilute solutions of potassium bromide. In one such experiment (Fig. 1), barley plants placed in a dark humid chamber had their roots immersed in aerated distilled water. They were decapitated about 1 inch above the root-stem plate and the bleeding sap collected for

<sup>1</sup> Hoagland, D. R. and Broyer, T. C., *Plant Physiol.*, 1936, **11**, 471.

<sup>2</sup> Prevot, P., and Steward, F. C., *Plant Physiol.*, 1936, **11**, 509.

<sup>3</sup> Steward, F. C., *Tr. Faraday Soc.*, 1937, **33**, 1006.

<sup>4</sup> Broyer, T. C., and Hoagland, D. R., *Am. J. Bot.*, 1940, **27**, 501.

15 minutes to obtain an initial sample of exudate. The experimental salt was then added; *i.e.*, KBr solution of 5 milliequivalents per liter concentration. Successive increments of exudate were collected for analysis. At several intervals root samples were removed, weighed, centrifuged, and frozen for the determination of the K and Br concentrations in the expressed sap. The data presented illustrate that the exudate, as well as the expressed sap of the roots, reflects the power of the actively metabolizing roots to concen-

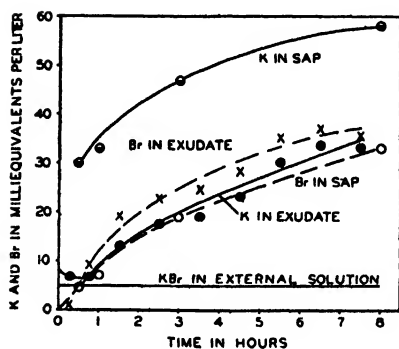


FIG. 1

FIG. 1. Comparison of the concentrations of K and Br in exudates and in expressed saps from roots of decapitated barley plants. Roots of decapitated plants in aerated distilled water (control) and in aerated KBr solution of 5 milliequivalents per liter concentration. The exuding plants were placed in dark, humid chambers.

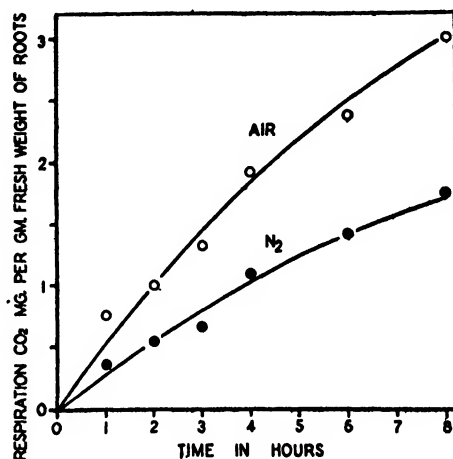


FIG. 2

FIG. 2. Comparison of rates of  $\text{CO}_2$  production by excised roots under aerobic and anaerobic conditions. Barley plants grown from Jan. 7th to Jan. 28th (21 days). Experimental culture solutions 50 milliequivalents per liter concentration. Temperature of culture solution  $19.0 \pm 0.5^\circ\text{C}$ . Nitrogen gas purified through pyrogallol. Roots placed in water and pretreated with  $\text{N}_2$  gas. Compare Figs. 3a and b, in which data are from a similar experiment.

trate solutes. Earlier investigators also observed and studied the relations between the composition of exudate fluids and that of the culture medium of the roots. In the recent period, this method of study has assumed greater interest in the light of developments in research on the active transport of solutes by living plant cells.

The data cited in this article indicate that the concentration of mobile ions may be of the same order of magnitude in the exudate and in expressed sap of the roots from which the exudate arises. They are in agreement with re-

sults published earlier from this laboratory.<sup>5</sup> Lundegårdh has recently cited from another point of view several results of similar import.<sup>6</sup> In the present experiments the bromide ion concentrations were nearly the same in the two fluids of different origin (exudate and expressed sap) after a suitable period of time for solute absorption had elapsed. Potassium concentrations remained considerably higher in the expressed sap than in the exudate. Undoubtedly the quantitative relations of solute concentration in root sap and exudate would vary with the time period of the experiment and with factors affecting metabolism. The idea to be stressed here is that the independent method of exudate study leads to essentially the same general point of view concerning the metabolically governed movement of salt against gradients as does the study of expressed sap. This is of interest because the exudate is a fluid obtained without the need of killing and crushing tissues. The exudate from the barley plants contained no protein precipitable by trichloroacetic acid. Phillis and Mason<sup>7</sup> have suggested that the accumulation of solutes by tissues of higher plants may be regarded as a phenomenon of the "solvent capacity" of living protoplasm. The evidence offered in this article, as well as the evidence available from the investigations of *Nitella*, *Valonia*, and other large coenocytic cells does not lead to the view that the activities of living plant cells in concentrating salt can be explained by the concept of "solvent capacity" of protoplasm. Rather a secretory process is indicated.

#### *Respiration in Relation to Salt Accumulation*

The intimate association of aerobic metabolism with inorganic solute accumulation by plant cells gives special importance to the processes of aerobic respiration. As a general basis for discussion of this aspect of the problem, it is useful to call attention to the rates of CO<sub>2</sub> production by barley root cells under aerobic and anaerobic conditions. Data from one experiment are presented in Fig. 2.<sup>8</sup> The aerobic CO<sub>2</sub> production is more rapid than the anaerobic, but even in the latter case enough carbonic acid is given off to make possible marked salt accumulation, if this were the determining factor.<sup>9</sup>

<sup>5</sup> Hoagland, D. R., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, 8, 181.

Hoagland, D. R., and Broyer, T. C., *Am. J. Bot.*, 1940, 27, 173.

<sup>6</sup> Lundegårdh, H., *Planta*, 1940, 31, 184.

<sup>7</sup> Phillis, E., and Mason, T. G., *Ann. Bot.*, 1940, 4, n.s., 645.

<sup>8</sup> Also see footnote 1 and data in Tables I and III.

<sup>9</sup> Unpublished data with excised barley roots show that a small aerobic accumulation of salt can occur at low temperature (0–5°C.) under which conditions the concomitant CO<sub>2</sub> production may be only 20 per cent of that at normal temperatures (20–25°C.) The rate of CO<sub>2</sub> production under anaerobic conditions is frequently 50 to 60 per cent of that under aerobic conditions, at similar, normal temperatures,

Yet anaerobic respiration is ineffective for this purpose. The respiratory quotient for well aerated cells is close to 1, provided an excess absorption of cation or anion does not occur. If an excess of an ion of one sign of charge is absorbed then through adjustments in organic acid metabolism the respiratory quotient may be altered (Ulrich).<sup>10</sup>

Because of the importance of aerobic respiration in relation to salt accumulation, it is an obvious step to investigate the influence on salt accumulation of respiratory inhibitors. A systematic study of roots from this point of view is going forward. In several experiments already completed potassium cyanide in relatively low concentration (0.5 to 10 milliequivalents per liter) was added to aerated solutions of potassium bromide in which barley roots were immersed. The cyanide suspended completely, or almost completely, the accumulation of K and Br ions, without apparent injury to the roots, except for a development of brown color in higher concentrations of cyanide (Table I, Experiment 1). The effect of cyanide on salt accumulation was to a certain extent reversible (Table I, Experiment 2). The lower effective concentrations of cyanide (0.5 to 1.0 milliequivalents per liter) did not depress CO<sub>2</sub> production very greatly, but the more significant oxygen consumption value may show greater depression, according to certain unpublished data by Machlis, of this laboratory. The results obtained with cyanide suggest an induced anaerobiosis. The general suggestion is that a metal catalyzed respiratory system is linked in some way with the process of salt accumulation, although preliminary experiments have not revealed the exact nature of the system involved.

It is conceivable that a hydrogen acceptor which would modify the course of respiration would likewise influence the accumulation of electrolytes. The effects of methylene blue were studied from this viewpoint. The presence of this hydrogen acceptor in the nutrient medium in very low concentrations ( $1 \times 10^{-5}$  to  $1 \times 10^{-4}$  molar) had little influence on either CO<sub>2</sub> production or electrolyte absorption. Methylene blue concentrations of  $1 \times 10^{-3}$  molar which were effective in inhibiting salt accumulation (Table II), did not depress the average rate of CO<sub>2</sub> production over an experimental period of 4 hours. Although the methylene blue-treated tissues ( $1 \times 10^{-3}$  M) frequently appeared to be turgid, a tissue injury was apparent in the general loss in fresh weight and solute by the roots during this interval of time. These effects were irreversible. Methylene blue increased the permeability of the cells, as indi-

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but appreciable salt accumulation does not occur. Since under low oxygen tensions the respiratory quotient increases, oxygen absorption would be a more satisfactory index of respiratory metabolism in relation to absorption of salt (compare data on respiration of plant tissues by Merry and Goddard, see Merry, J., and Goddard, D. R., *Proc. Rochester Acad. Sc.*, 1941, 8, 28) than CO<sub>2</sub> produced.

<sup>10</sup> Ulrich, A., *Am. J. Bot.*, 1941, 28, 526.

TABLE I

*Effects of Aeration and of Cyanide on the Absorption of K and Br by Excised Barley Roots*

Experimental conditions	Composition of expressed sap per liter		Net absorption per liter		Respiration rate in amount of CO <sub>2</sub> per hr. per gm. fresh weight tissue
	K	Br	K	Br	
	m. eq.	m. eq.	m. eq.	m. eq.	mg.
<i>Experiment 1</i>					
Control roots (initial status) . . . . .	26.8	0.0			
Distilled water; aerated . . . . .	25.2	0.0	-1.6	0.0	0.29
Distilled water; N <sub>2</sub> gas . . . . .	21.7	0.0	-5.1	0.0	0.16
KBr 5 m. eq. per l.; aerated. . . . .	55.9	22.3	29.1	22.3	0.32
KBr 5 m. eq. per l.; N <sub>2</sub> gas. . . . .	26.7	3.2	-0.1	3.2	0.12
KCN 0.5 m. eq. per l.; aerated . . . . .	25.6	0.0	-1.2	0.0	0.29
KCN 1.0 m. eq. per l.; aerated . . . . .	22.5	0.0	-4.3	0.0	0.20
KCN 3.0 m. eq. per l.; aerated . . . . .	27.3	0.0	0.5	0.0	0.08
KBr 5 m. eq. per l., plus KCN 0.5 m. eq. per l.; aerated . . . . .	26.4	4.7	-0.4	4.7	0.27
KBr 5 m. eq. per l., plus KCN 1.0 m. eq. per l.; aerated . . . . .	24.3	0.0	-2.5	0.0	0.19
KBr m. eq. per l., plus KCN 3.0 m. eq. per l.; aerated . . . . .	31.8	0.8	5.0	0.8	0.10
<i>Experiment 2</i>					
Control roots (initial status) . . . . .	18.6	0.0			
KBr 5 m. eq. per l. . . . .	44.5	33.8	25.9	33.8	
KBr 5 m. eq. per l., plus KCN 10.0 m. eq. per l. . . . .	23.9	2.1	5.3	2.1	
KBr 5 m. eq. per l., plus KCN 10.0 m. eq. per l., followed by washing, then KBr 5 m. eq. per l. . . . .	31.9	18.0	13.3	18.0	
<i>Experiment 3</i>					
Control roots (initial status) . . . . .	25.0	0.0			
KBr 50 m. eq. per l., plus KCN 1.0 m. eq. per l. . . . .	29.2	6.8	4.2	6.8	

Experiment 1: Barley plants grown from Oct. 15 to Nov. 8 (24 days). Experimental periods 8 hours. pH of cyanid cultures about 7.0. Nitrogen gas purified through pyrogallol. Temperature of culture solutions 22°C.

Experiment 2: Barley plants grown from June 3 to June 24 (21 days). Experimental periods each 5 hours. All culture solutions aerated. Temperatures of culture solutions 21.5 ± 0.5°C.

Experiment 3: Barley plants grown from Dec. 30 to Jan. 30 (31 days). Experimental periods 8 hours. Culture solution aerated. Temperature of culture solution, 17.0 ± 0.5°C.

cated by outward movement of solutes, and CO<sub>2</sub> production continued, but disturbance of the protoplasmic mechanism prevented salt accumulation.



The influence of oxygen deficiency in preventing salt accumulation might be associated with the formation of by-products of fermentative processes, such as alcohol or lactic acid. An experiment was carried out in which these substances were added to the culture solution, under aerobic and anaerobic conditions (Table III). Under aerobic conditions neither the alcohol (0.01 M) nor the lactic acid (0.001 M) had the inhibiting effect on the entrance of salt into the tissue that was produced by oxygen deprivation. It may be that oxidation of the lactic acid or alcohol was sufficiently rapid to prevent any

TABLE II  
*Effects of Methylene Blue on the Absorption of K and Br by Excised Barley Roots*

Experimental conditions	Composition of expressed sap per liter		Net absorption per liter		Respiration rate in amount of CO <sub>2</sub> per hr. per gm. fresh weight tissue
	K	Br	K	Br	
	m. eq.	m. eq.	m. eq.	m. eq.	
<i>Experiment 1</i>					mg.
Control roots (initial status) . . . . .	22.3	0.0			
Distilled water . . . . .	21.0	0.0	-1.3	0.0	0.29
Methylene blue 0.001 M . . . . .	8.7	0.0	-13.6	0.0	0.32
KBr 5 m. eq. per l. . . . .	37.9	7.85	15.6	7.85	0.39
KBr 5 m. eq. per l. plus methylene blue 0.001 M . . . . .	12.5	2.04	-9.8	2.04	0.43
KBr 5 m. eq. per l. plus methylene blue 0.001 M, followed by wash- ing, then KBr 5 m. eq. per l. . . . .	11.8	4.05	-10.5	4.05	0.26*

Experiment 1: Barley plants grown from Apr. 8 to May 2 (24 days). Experimental periods each 4 hours. Culture solutions aerated. Temperatures of culture solutions  $21.5 \pm 0.5^\circ\text{C}$ . At the termination of each experimental period all roots appeared to be turgid; losses in fresh weight of tissue during the experiment were not significant. Sugar losses from roots treated with methylene blue were significantly greater than those accountable to normal respiratory processes.

\* This figure represents the respiration rate obtained during the secondary treatment. The value during the primary treatment was 0.40.

interference with protoplasmic reactions. Or, as Blinks<sup>11</sup> suggests, on the basis of the work of Osterhout and Hill,<sup>12</sup> it is conceivable that under anaerobiosis there may exist as an influential factor, not the formation of a deleterious product of metabolism, but rather the failure of synthesis of some protoplasmically active compound.

<sup>11</sup> Blinks, L. R., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, 8, 204.

<sup>12</sup> Osterhout, W. J. V., and Hill, S. E., *Proc. Soc. Exp. Biol. and Med.*, 1934-35, 32, 715.

Another approach to the possibility that absorption of salt might be retarded or prevented through by-products of fermentative processes was made. It was considered that these by-products might not be formed in significant quantities anaerobically under conditions of low temperature. If permeability *per se* were not impaired or not essentially low at all times, then ions should move readily into the cell *with* a positive inward concentration gradient at a low temperature. In one experiment, the results of which are presented in Table IV, the effects of passing air and N<sub>2</sub> gas through the culture solution on the absorption of K and Br with a concentration gradient were compared.

TABLE III

*Effects of Lactate and of Ethanol on the Absorption of K and Br by Excised Barley Roots*

Experimental conditions	Composition of expressed sap per liter		Net absorption per liter		Respiration rate in amount of CO <sub>2</sub> per hr. per gm. fresh weight tissue
	K	Br	K	Br	
	m. eq.	m. eq.	m. eq.	m. eq.	mg.
Control roots (initial status).....	23.2	0.0			
KBr 50 m. eq. per l., plus K lactate 0.001 M (pH 5); aerated.....	77.1	38.7	53.9	38.7	0.33
KBr 50 m. eq. per l., plus K lactate 0.001 M (pH 5); N <sub>2</sub> gas.....	27.9	8.1	4.7	8.1	0.19
KBr 50 m. eq. per l., plus ethyl alcohol 0.01 M, aerated.....	73.0	33.5	49.8	33.5	0.34
KBr 50 m. eq. per l., plus ethyl alcohol 0.01 M; N <sub>2</sub> gas.....	16.9	7.9	-6.3	7.9	0.19

Barley plants grown from Aug. 28 to Sept. 18 (21 days). Experimental periods 12 hours. Temperature of culture solutions  $23.5 \pm 0.5^\circ\text{C}$ . Nitrogen gas purified through pyrogallol. All tissues were turgid at the termination of the experiment. Fresh weights and sugar contents of tissues were maintained. Roots placed in water and pretreated with N<sub>2</sub> gas.

The extremely slow entry of those ions, either at the lower or the higher temperature, when N<sub>2</sub> gas was passed, does not supply evidence for a temperature controlled effect on the formation of substances influencing permeability. However, the modifying influence of low temperatures on the viscosity of protoplasm and membrane structure must not be disregarded.

Thus far, except for the temperature experiment just discussed, we have had in mind studies of salt accumulation from dilute solutions (5 milliequivalents per liter or less) in which the initial concentration of potassium in the culture solution was much lower than the initial concentration of K in the roots. Bromide although not initially present in the roots, rapidly rose to a concentration higher than that of the external solution, and the potassium was concomitantly accumulated against the existent concentration gradient. Other

experiments have now been made with solutions of much higher concentration of K and Br in the culture medium, but not so high as to induce harmful osmotic disturbances. These concentrations were 50 or 60 milliequivalents per liter of KBr. The inward gradient of K was then positive from the beginning.

Effects on inward salt movement were compared for aerated solutions and solutions through which purified nitrogen gas was passed (Figs. 3*a* and 3*b*). Anaerobically very little potassium entered the roots, notwithstanding that a marked inward gradient existed for this ion, while aerobically the potassium was rapidly absorbed and even accumulated to a higher concentration than that of the relatively strong external solution. Bromide ions moved inward

TABLE IV

*Effects of Temperature and Aeration on the Absorption of K and Br by Excised Barley Roots*

Experimental conditions	Composition of expressed sap per liter		Net absorption per liter	
	K	Br	K	Br
	<i>m. eq.</i>	<i>m. eq.</i>	<i>m. eq.</i>	<i>m. eq.</i>
Control roots (initial status).....	22.0	0.0		
KBr 60 m. eq. per l., 0.75°C; aerated.....	40.5	16.1	18.5	16.1
KBr 60 m. eq. per l., 0.75°C; N <sub>2</sub> gas .....	27.8	9.7	5.8	9.7
KBr 60 m. eq. per l., 18.5°C; aerated.....	80.4	50.0	58.4	50.0
KBr 60 m. eq. per l., 18.5°C; N <sub>2</sub> gas.....	29.2	10.7	7.2	10.7

Barley plants grown from Nov. 4 to Nov. 27 (23 days). Experimental periods 12 hours. Nitrogen gas purified through pyrogallol. Temperature of culture solutions  $0.75 \pm 0.05^\circ\text{C}$ . and  $18.5 \pm 0.5^\circ\text{C}$ . The specific conductivities of the expressed sap followed directly the changes in K and Br. Roots placed in water and pretreated with N<sub>2</sub> gas.

only to a slight degree when the cells were deprived of oxygen, but entry was rapid when the cells were adequately aerated. When CO<sub>2</sub> was passed through the culture solution, the absorption curves for K and Br were similar to those for N<sub>2</sub> gas (Figs. 3*a* and 3*b*). Further, it is apparent from the data of Table I, Experiment 3, that the suppression of entry of KBr into the cells even with an inward concentration gradient, under aerobic, cyanide treatment, was likewise similar to the anaerobic effect obtained when oxygen was withheld (N<sub>2</sub> gas treatment). Incidentally, attention may be called to the relative rates of aerobic accumulation of potassium and bromide ions (Fig. 3*a*). As frequently happens in the early stages of salt absorption by roots of high absorbing capacity, potassium ions were absorbed more rapidly than the associated anions. This involves ion exchanges which have been discussed elsewhere.<sup>13</sup>

<sup>13</sup> Broyer, T. C., and Overstreet, R., *Am. J. Bot.*, 1940, 27, 425.

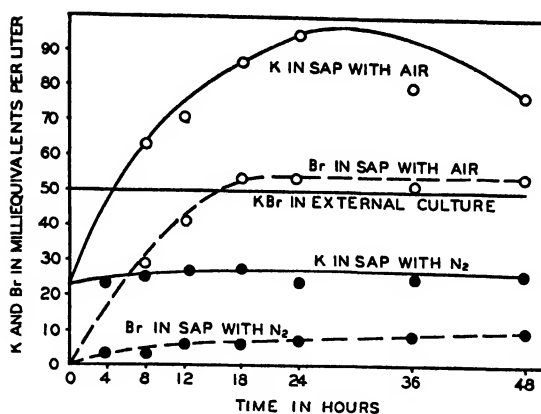


FIG. 3a

FIG. 3a. Effects of air and  $N_2$  on the absorption of K and Br by excised barley roots, under conditions of an inward concentration gradient.

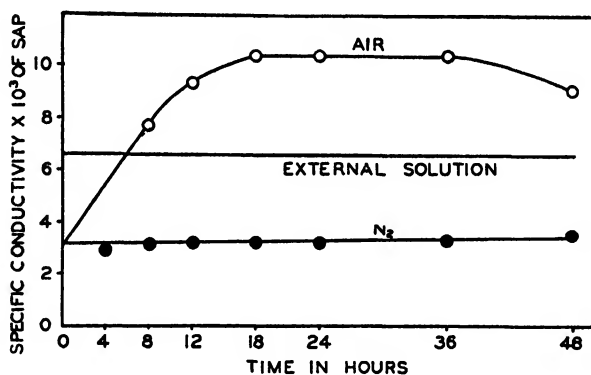


FIG. 3b

FIG. 3b. Effects of air and  $N_2$  on the absorption of K and Br by excised barley roots, under conditions of an inward concentration gradient, as measured by electrical conductivity.

FIGS. 3a and 3b. Barley grown from June 23 to July 15 (22 days). Temperature of culture solutions  $22.0 \pm 0.5^\circ\text{C}$ . Nitrogen gas purified through pyrogallol. Roots placed in water and pretreated with  $N_2$  gas. Roots in 48 hour aerated condition were slightly flaccid at time of removal; roots of all other sets were turgid. Compare Fig. 2, which data are from a similar experiment.

Under an anaerobic environment the roots may lose to distilled water a small amount of potassium. This does not occur to a significant degree under an aerobic environment, as long as cells remain in healthy condition. The outward movement of ions under anaerobiosis, over limited experimental

periods, is nevertheless slight in comparison with the loss from markedly injured cells, and sometimes it is not certain that any net efflux occurs. When it does, it is still possible that some, if not all, of the loss may have originated in senescent cells, breaking down more rapidly because of oxygen deficiency. Conceivably many of the cells retain their solutes completely for a considerable period even though they are unable to accumulate more solutes. Undoubtedly over a long period of time oxygen is essential to maintain barley root cells in a state enabling them to retain their solutes, but they can maintain without oxygen supply a salt concentration higher inside than outside for 48 hours or more.

The data already discussed raise various questions of permeability, in the restricted sense of the term, *versus* active transport of salt or ions. The extensive work of Blinks<sup>14</sup> on *Halicystis* cells requires attention in this connection. He studied the effects of oxygen on bioelectric potentials and in relation to ionic discrimination by the protoplasm. The effects of oxygen deprivation of sufficient degree in decreasing or abolishing the normally maintained bioelectrical potential, were very striking. Over a considerable period of time, the effects were reversible. This investigator also measured electrical resistance across the protoplasm (by a direct current method) of cells supplied with oxygen in comparison with cells deprived of oxygen. The resistance was found to be far greater for the cells deprived of oxygen. These observations were interpreted in terms of decrease in permeability of some protoplasmic surface or decrease of mobility of ions in the protoplasm in general, when aerobic metabolism is abolished.

The data we have cited on root tissues could likewise be considered as indicating a greatly decreased cell permeability as a consequence of anaerobiosis, or of the action of respiratory poisons like cyanide. On the other hand, at least as far as the root experiments are concerned, one may conceive that a pumping mechanism is made inoperative when some component of the aerobic respiratory system fails under oxygen deficit, or when an inhibitor of aerobic respiration is in action. In other words, the cells may be relatively impermeable, at least at the vacuolar membrane, under these circumstances (until protoplasmic breakdown occurs) as well as when active aerobic metabolism is taking place, but the latter process permits the solute to be actively secreted, as a net effect, into the vacuole. There would still remain for elucidation the ionic exchanges that can take place, especially when anions are involved. (Compare early observations on Br-Cl exchanges in *Nitella*.<sup>15</sup>) On this point it may be noted, however, that certain experiments on roots with

<sup>14</sup> Blinks, L. R., Darsie, M. L., Jr., and Skow, R. K., *J. Gen. Physiol.*, 1938, **22**, 255.

<sup>15</sup> Hoagland, D. R., Hibbard, P. L., and Davis, A. R., *J. Gen. Physiol.*, 1926, **10**, 121.

radioactive bromide indicate that while considerable exchange of radioactive for non-radioactive bromide ions can occur, aerobically, (data on anaerobic conditions are not completed) at the same time the net inward movement of bromide greatly surpasses the exchange of ions.

Collander<sup>16</sup> has shown in experiments on *Chara* and *Tolypellopsis* cells that the exchange of cations between sap and solution is extremely slow and he believes that this means a high degree of impermeability in the protoplasm or its membranes. Overstreet and Broyer<sup>17</sup> have published data on root cells indicating that only about 10 per cent of the potassium present in the cells is readily exchangeable in experiments with isotopes. It is not certain that even this limited exchange occurs between vacuole and solution. About 10 per cent of the potassium is not recoverable in composite expressed sap.<sup>18</sup>

One rather crude concept would be that the salt or its ions are forced into the vacuole, although membrane properties prevent them from readily diffusing out into distilled water. Another concept is that of a dynamic system in which the salt or ions of the salt are actively transported to the vacuole and the solutes moving outward are carried back, except as certain ionic exchanges meanwhile take place. We are, of course, aware that these speculative views imply merely that the mechanism of salt accumulation remains an unsolved problem. In any event, the system of the higher plant as a whole involves other complications. Salt previously accumulated in root cells may be removed inward and then be carried upward to be accumulated by cells of the shoot. This polarized movement out of root cells also requires much further investigation. (Compare Crafts and Broyer.<sup>19</sup>)

### *Experiments on Nitella Cells*

Additional light on some of the problems of salt accumulation comes from recent experiments on *Nitella* cells in which radioactive isotopes were employed. Brooks<sup>20</sup> was the first to apply this technique to *Nitella* cells. A rough separation of cell sap, protoplasm, and cell wall was made. Brooks found that radioactive rubidium entered the protoplasm rapidly, although subsequent movement into the vacuole was very slow under the conditions of his experiments. The metabolic activity and capacity for accumulating additional total salt are at a far lower rate level in *Nitella* than in roots of the type referred to in this article. We have recently conducted other experiments on *Nitella* with radioactive isotopes, to supplement as directly as possible the experiments on roots and to carry further early studies on *Nitella* in this laboratory.<sup>21</sup>

<sup>16</sup> Collander, R., *Protoplasma*, 1939, **33**, 215.

<sup>17</sup> Overstreet, R., and Broyer, T. C., *Proc. Nat. Acad. Sc.*, 1940, **26**, 16.

<sup>18</sup> Broyer, T. C., and Hoagland, D. R., *Am. J. Bot.*, 1940, **27**, 501.

<sup>19</sup> Crafts, A. S., and Broyer, T. C., *Am. J. Bot.*, 1938, **25**, 529.

<sup>20</sup> Brooks, S. C., *J. Cell. and Comp. Physiol.*, 1938, **11**, 247.

<sup>21</sup> Hoagland, D. R., and Davis, A. R., *Protoplasma*, 1929, **6**, 610.

*Nitella* cells were grown in pond water. Nodal cells and the terminal bud were removed (in Experiments 1 and 2 to be cited) from the main axis of the thallus. The large internodal cells were replaced into vessels containing pond water to allow them to reestablish equilibrium with their normal environment. These filaments (or in Experiment 3, untreated group of cells) were placed into solutions containing radioactive salt and subjected to either continuous light or darkness, with air or N<sub>2</sub> gas continuously passed through the bathing medium. The light intensity, when applied, was 200 watts (daylight, fluorescent lamps) at approximately 2 feet. Large internodal cells were individually broken and the vacuolar sap collected. This was centrifuged and an aliquot of the clear supernatant sap counted for its radioactivity. After removing the sap, the residues from the debranched filaments, including protoplasm and cell walls (Brooks has shown the latter to possess little or no capacity for accumulation) were dried and leached to volume, with hot acidulated water, to remove the radioactive ions sought. Counts on aliquots of the filtered leach were converted for tabular comparison to activity per milliliter of residual sap, based on the loss in weight on drying the residue.

We shall refer here only to several experiments with radioactive rubidium or bromide ions. The data now at hand give evidence in accordance with the conclusion of Brooks, that at first there is an accumulation of the radioactive ions in the protoplasm, but the present data suggest continued movement of the ions into the vacuole (Table V, Experiment 1) until in course of time the concentration in the vacuole (at least for Br) exceeds that in the protoplasm (Table V, Experiment 2). If this latter relation can be substantiated,<sup>22</sup> it would imply a secretory process, operating from protoplasm to vacuole. Under an aerobic condition, the radioactive ions accumulated in the vacuole to a definitely higher concentration than that in the external solution, but this concentrating effect did not appear under an anaerobic condition, which was attained by placing cells in the dark with N<sub>2</sub> gas passed through the solution (Table V, Experiment 3). Cells in the light, of course, produce oxygen and can accumulate salt, even if no air is passed through the solution, although there is a suggestion from one experiment that aerated cells in the light have the greatest activity for the inward movement of salt. Preliminary data by Blinks<sup>23</sup> further suggest that entry of ions into the vacuoles of *Halicystis* cells is influenced by oxygen supply. In an aerobic environment radioactive rubidium entered the cells until vacuolar sap concentration finally exceeded protoplasmic concentration.

Some of the evidence on *Nitella* cells from the work of Brooks and others

<sup>22</sup> At present it is not possible to evaluate the errors resulting from contamination of cell residue by external solution or vacuolar sap. Contamination would also be present if attempts were made to remove protoplasm according to the technique of Brooks.

<sup>23</sup> Private communication.

may mean that the vacuolar membrane has but very slight permeability. Nevertheless, by the pump-like action, additional increments of salt may move into the vacuole under sufficiently favorable metabolic conditions. We believe that movement of this kind is particularly fast in actively metabolizing root cells. Certainly if the root system as a whole is considered, very rapid accumu-

TABLE V  
*Absorption of Rb\* and Br\* Ions by Nitella*

Experimental conditions	Br* in counts per min. per ml.			Br* ratios in counts per min. per ml.			Spec. cond. in mhos $\times 10^{-3}$	
	Sap		Ex- ternal solution	Vacuole	Vacuole	Residue	Vacuo- lar sap	Ex- ternal solution
	Vacuole	Residue		Residue	Ex- ternal solution	Ex- ternal solution		
<i>Experiment 1</i>								
Rb*Cl aerated, light 1 day	82	417	33	0.2	2.5	12.6		
Rb*Cl aerated, light 10 days.	400	545	33	0.7	12.1	16.5		
<i>Experiment 2</i>								
Control cells (initial status)...							6.9	
KBr* aerated, light 23 hrs. . .	5180	3840	280	1.4	19	14	8.9	0.2
KBr* aerated, light 71 hrs. . .	12000	6750	280	1.8	43	24	10.4	0.2
<i>Experiment 3</i>								
Rb*Cl, aerated, light . . . . .	74	—	30	—	2.5	—		
Rb*Cl, N <sub>2</sub> gas, dark . . . . .	23	—	24	—	0.7	—		

Experiment 1: *Nitella* cells from pond water. Nodal cells and terminal buds removed from cells of main axis of thallus. Only these latter cells were used in the absorption studies. The experimental external solution was Rb\*Cl 1.0 milliequivalent per liter.

Experiment 2: *Nitella* cells from pond water. Nodal cells and terminal buds removed from cells of main axis of thallus. Only these latter cells were used in the absorption studies. The experimental external solution was KBr\* 1.18 milliequivalents per liter.

Experiment 3: *Nitella* cells from pond water. A composite group of cells was used during the absorption period. Vacuolar sap was obtained from large cells of the composite sample. The experimental external solution was Rb\*Cl 5 milliequivalents per liter.

lation of salt in the root exudate can take place. Within less than half an hour mobile ions can be transferred to the exudate in concentrations higher than those of the solution from which absorption takes place.

#### *Studies on Fluids Obtained by Suction from Tomato Roots*

Another way of attacking the general problem will now be considered. Sturdy young tomato plants were decapitated several inches above the junction of stem and root and by attaching a tube to the cut stem, suction was applied



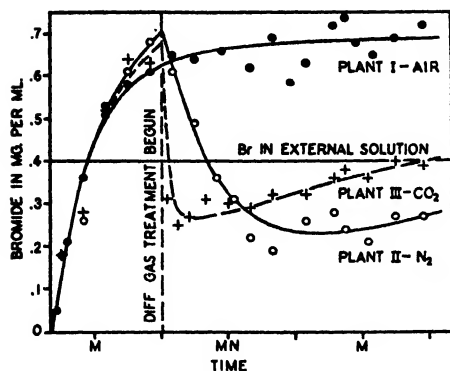


FIG. 4a

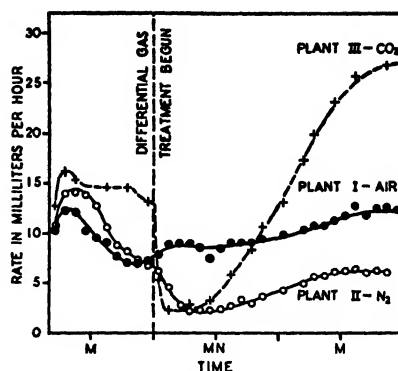


FIG. 4b

FIG. 4a. Effects of differential gas treatments on the concentration of bromide ions in exudates from decapitated tomato plants.

FIG. 4b. Effects of differential gas treatments on the rate of exudation by decapitated tomato plants.

FIGS. 4a and 4b. Tomato plants grown with aeration in a culture solution of the following composition:

KNO <sub>3</sub> .....	0.0025 M
Ca(NO <sub>3</sub> ) <sub>2</sub> .....	0.0025 M
MgSO <sub>4</sub> .....	0.001 M
KH <sub>2</sub> PO <sub>4</sub> .....	0.0005 M
Fe as iron tartrate; 1 ml. of 0.5 per cent solution per liter of culture solution.	

Micronutrients as required.

Growth period from Oct. 10 to Nov. 26 (47 days). Experimental culture solution 5 milliequivalents per liter. Gases were continuously passed through the solutions. All roots were aerated until 6 P.M. Differential gas treatments of air, N<sub>2</sub> and CO<sub>2</sub> were then applied. Suction equal to 740 mm. Hg was continuously applied (except during observations on volume of exudate and during removal of increments of exuded fluid) throughout the experiment. At the termination of the experiment the abilities of the plants to develop an exudation pressure (as a measure of the state of the root systems) were noted. The following results were obtained: aerated cultures, appreciable and maintained exudation pressure; N<sub>2</sub> treated cultures, a weak yet maintained pressure; CO<sub>2</sub> treated cultures, no exudation pressure over an extended interval of time. The roots in the CO<sub>2</sub> treated culture were flaccid at the termination of suction exudation.

and the fluid obtained examined under various conditions in the root environment. This is essentially the method described by Kramer.<sup>24</sup>

The results of one experiment are presented in two graphs (Figs. 4a

<sup>24</sup> Kramer, P. J., *Am. J. Bot.*, 1940, 27, 216.

and 4 *b*). The first sections of the curves represent data obtained on fluid recovered in the period during which air was passed through the solution in contact with the roots. Then one plant continued to receive aeration, one was subjected to nitrogen gas, and one to carbon dioxide gas. In the two latter cases, an abrupt downward trend in the concentration curves for bromide is evident (Fig. 4 *a*). The concentration of this ion in the recovered fluid became much lower than that of the external solution, whereas during the aerobic period it was much higher. There followed an upward trend in the CO<sub>2</sub> curve until the concentration of bromide attained approximate equality with that of the solution. The upward trend of the N<sub>2</sub> curve, within the time period of the experiment, was less marked and the concentration of bromide in the fluid recovered by suction remained lower than that of the external solution. The other figure (Fig. 4 *b*) represents volumes of solution recovered over various time intervals (compare Kramer). Similar trends in the curves for the several treatments are observable. The injurious effect of the CO<sub>2</sub> treatment was manifest in the latter stages of the experiment, but in the nitrogen-treated plants similar effects were much less apparent.

The most obvious interpretation of these experimental results is that the effects of N<sub>2</sub> and CO<sub>2</sub> cause initially a decrease in root permeability to inorganic solutes and to water, and also in power to accumulate solutes; followed by a protoplasmic breakdown with increase of permeability, *without* power of solute accumulation on the part of the cells.

#### DISCUSSION

If all the various lines of evidence are brought together, the general picture would seem to be that permeability, metabolism, and accumulation of salt are so intimately interrelated that generally it becomes impossible to disentangle the several aspects of the phenomena. Blinks has emphasized, on the basis of his bioelectric measurements on *Halicystis* cells that permeability and metabolism may be connected through the relation of metabolism to the formation and maintenance of protoplasmic membranes. As we have already pointed out, in the root systems of plants there are still other reactions to explain in the polarized movement of ions out of the root cells. As the subject develops and the results of new types of experiments become available, the general conclusion is reinforced that the absorption and movement of ions in the plant, in nearly all their aspects, require an increasing understanding of the metabolism of plant cells. The limitations of many earlier experiments on permeability of cells in their application to problems of plant nutrition become clear. It has seemed especially appropriate to submit this brief discussion on accumulation of inorganic solutes and permeability in plant cells for publication in a volume dedicated to Dr. Osterhout, who has for so many years contributed to this field of inquiry. The article has been prepared with the

hope that the data presented may serve a purpose as an aid to stimulating further discussion of the problems of plants absorbing their inorganic nutrients from dilute media.

#### SUMMARY

1. Comparisons are made of concentrations of K and Br in exudates of barley roots and in expressed sap from roots, under conditions favorable for aerobic metabolism. Both methods lead to the same general viewpoint concerning metabolically governed transport of solutes by living plant cells.

2. Cyanide in low concentration prevented salt accumulation by barley roots. Methylene blue, without decrease of CO<sub>2</sub> production by roots, destroyed power of salt accumulation.

3. K and Br ions entered roots to only a slight extent under an anaerobic condition, even with an inward gradient of ionic concentration.

4. Lactate or alcohol, under aerobic conditions, did not prevent rapid accumulation of salt by root cells.

5. Experiments on fluids obtained by suction from tomato roots gave evidence of loss of salt-accumulating power under the influence of N<sub>2</sub> gas or CO<sub>2</sub> gas, together with probable effects on cell permeability.

6. Several experiments on *Nitella* cells in which radioactive isotopes were used are reported. Bromide gradually moved into vacuolar sap until the concentration appeared to exceed that of the protoplasm, on the basis of the results of the several types of experiments. Accumulation of salt in the vacuole did not occur anaerobically.

7. Some views of interrelations of permeability, salt accumulation, and metabolism are suggested for further discussion.

# THE PREPARATION AND USE OF TOBACCO MOSAIC VIRUS CONTAINING RADIOACTIVE PHOSPHORUS

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PLATES 6 AND 7

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One of the major mysteries surrounding viruses is their manner of reproduction or growth. Despite the fact that the introduction of one unit of a virus within a living cell of a susceptible host is followed by the production of millions of virus units, practically nothing is known concerning the reproductive process. It is not known whether subsequent generations of virus are produced by some catalytic process in which none of the substance of the inoculum is incorporated into subsequently produced virus, or by a process similar to that involved in the growth of organisms in which a portion of the substance of the parent is transferred to the progeny, or by means of some new and unknown process. This problem is one of considerable importance, for it is related directly to the fundamental nature of viruses. It seemed possible that the preparation and isolation of tobacco mosaic virus containing radioactive phosphorus, the inoculation of plants with virus marked by radioactivity, and the determination of the distribution of the radioactivity in the diseased plants following extensive multiplication of virus should provide some measure of information concerning the process of virus reproduction. If, following the inoculation of the marked virus, radioactivity should be demonstrable in portions of the plant other than in the inoculated leaves, it might provide a basis for assuming that reproduction by fission had occurred. If, on the other hand, all of the radioactivity in the inoculum could be recovered from the inoculated leaves, the result might be interpreted to mean that reproduction had followed some catalytic process.

There is evidence that tobacco mosaic virus in mosaic-diseased plants acts as a foreign protein in that it does not appear to enter into catabolic processes and cannot be utilized in the synthesis of normal plant proteins (1, 2). It seemed possible, therefore, that theoretical objections to the method of approach outlined above, based on the known lability *in vivo* of components of proteins (3), might not necessarily prove valid in the present case. Although rapid movement of virus within plant stems has been noted (4), it is possible that a given unit of virus usually remains localized and does not move from place to place. Despite the number of theoretical and experimental difficulties, including those just mentioned and those discussed by Arnon, Stout, and

Sipos (5), it appeared worthwhile to perform a few experiments in an attempt to determine the feasibility of this general method of approach to the question of the nature of virus reproduction.

In the present study, both normal and mosaic-diseased Turkish tobacco plants were fed a nutrient solution containing radioactive phosphorus and, after suitable periods of time, the distribution of radioactivity was determined in each set of plants. The tobacco mosaic virus which was present in the diseased plants was isolated and found to contain radioactive phosphorus. After purification, this virus was used as an inoculum for normal Turkish tobacco plants and the distribution of radioactivity and virus in inoculated and uninoculated portions of these plants was determined after 12 days. Although virus containing radioactive phosphorus was isolated from uninoculated portions of the plants, the results must be regarded as inconclusive because of reasons to be discussed later.

#### EXPERIMENTAL

The author is greatly indebted to Dr. John Lawrence and the Staff of the Radiation Laboratory of the University of California for the radioactive phosphorus that was used in the present experiments. The author also wishes to thank Dr. Robert Wilson and Mr. Ernest Frank of Princeton University for testing the samples obtained in the present work for radioactivity. These tests were made by placing each of the dried samples a given distance from a Lauritsen type electroscope or from the tube of a Geiger-Müller counter (5, 6).

A preliminary experiment was made in order to provide information regarding the nature and the rapidity of absorption of radioactive phosphorus by mosaic-diseased Turkish tobacco plants. A solution containing 51 mg. of disodium phosphate and 0.25 millicuries of radioactivity was added to 4 pots containing medium-sized mosaic-diseased Turkish tobacco plants which had been grown in sand and fed with a complete nutrient solution as described by Spencer (1). After 48 hours, the plants were removed, their roots well washed, and the entire plants frozen. The wash water from the roots and washings from the sand were combined and evaporated to dryness. The frozen plants were macerated and the virus was isolated by ultracentrifugation according to the method customarily employed in this laboratory (7). The various fractions into which the radioactivity might have been partitioned, namely, the washings from the sand and roots, the mash or press cake, the supernatant liquid obtained on ultracentrifugation, and the isolated virus, were then incinerated and on being tested were found to contain radioactivity in the ratios of 60, 12.7, 26, and 1.3, respectively. Thus, the major portion of the radioactive phosphorus which had been made available to the mosaic-diseased plants had not been taken up by the plants during the 48 hour period, and of that absorbed only 3.2 per cent was incorporated in the virus protein. It

seemed likely, therefore, that, in order to secure virus possessing a reasonable amount of radioactivity, it would be necessary to feed radioactive phosphorus to mosaic-diseased plants over a considerable period of time.

In the next experiment, 12 seedling Turkish tobacco plants were started in sand and 12 days later 6 of the plants were inoculated with tobacco mosaic virus. Then each of 3 normal and 3 diseased plants from this group was fed 50 ml. of a nutrient solution prepared by adding 330 mg. of disodium phosphate containing 7 millicuries of radioactivity to 20 liters of the complete nutrient solution described by Spencer (1). The remaining diseased and normal plants were fed a nutrient solution prepared by adding 330 mg. of ordinary disodium phosphate to 20 liters of the complete nutrient solution. The nutrient solutions were added daily in amounts which were increased from 50 to 200 ml. per plant, so that during the 30 day feeding period approximately the same amount of radioactive phosphorus was added each day.

Following the feeding period, the 4 sets of plants were watered freely with tap water for 20 days. Some idea of the distribution of radioactivity after different periods of time may be gained from the contact "radiographs" shown in Figs. 1 and 2. These were prepared by the technique described by Arnon, Stout, and Sipos (5), except that the leaves were not detached from the plants and an exposure period of about 12 hours was used. It may be seen that there was considerable radioactivity dispersed throughout both normal and diseased leaves at the end of 1 week and that there does not appear to be much difference in appearance between the radiographs of the normal leaves and those of the diseased leaves. Most of the radioactivity appears to have been distributed in the major veins of the leaves. Although the light green areas of diseased leaves are known to contain much more virus than the dark green areas (8), there does not appear to have been a concentration of radioactivity in the light green areas. Because of the high concentration of virus nucleoprotein known to exist in diseased leaves (7), it might be expected that such leaves would contain more phosphorus than normal leaves. However, when leaves were removed from each of the 4 sets of plants described above and dried at 110°, the dried leaves from normal plants fed radioactive and ordinary phosphorus were found to contain 0.94 and 0.78 per cent, respectively, of phosphorus, whereas the corresponding diseased leaves were found to contain 0.91 and 0.78 per cent, respectively. Comparable results were obtained with similar sets of plants as well as with plants grown in soil. It is obvious that there is no significant difference between the total phosphorus contents of normal and of mosaic-diseased Turkish tobacco leaves.

At the end of 50 days, the 4 sets of plants were cut, weighed, and placed in a room held at -12°. The sets of normal plants fed ordinary and radioactive phosphorus weighed 590 gm. and 621 gm., respectively, whereas the

corresponding sets of diseased plants weighed 430 gm. and 385 gm., respectively. The roots, sand, and pots from the 2 sets of plants fed radioactive phosphorus were well washed and the wash waters concentrated and saved. The virus present in the diseased plants which had been fed with radioactive phosphorus was then isolated by means of differential centrifugation. The water-insoluble material which was obtained on ultracentrifugation was added to the mash or press cake. The supernatant liquids which were obtained

TABLE I  
*Distribution of Radioactivity in Normal and Tobacco Mosaic-Diseased Plants\**

Experiment No.	Virus	Supernatant liquids from first 3 ultracentrifugations		Supernatant liquid from 4th ultracentrifugation	Press cake plus insoluble material from ultracentrifugation	Wash of sand and roots
		Protein	Non-protein			
1† (diseased)	12.3	14.6	27.0	0	21.7	24.4
2§ (diseased)	20.7	7.2	20.3	0	21.0	30.8
2§ (normal)		17.6	27.2		29.6	25.6

\* The radioactivity was measured by means of a Lauritsen type electroscope. In some cases readings were also made by means of a Geiger-Müller counter. The numbers represent the per cent of radioactivity found in the various fractions obtained from a given set of plants.

† In this experiment the virus fraction was dried from the frozen state and tested. All other fractions were incinerated and tested.

§ In this experiment, preliminary tests were made on fractions prepared as in Experiment 1. However, the numbers given refer to tests made on the fractions following precipitation of phosphorus by ammonium molybdate. This was done to reduce self-absorption due to the great bulk of some of the samples. Nevertheless, for reasons unknown, the radioactivity estimated to have been recovered was only about one-fifth the amount that was estimated to have been present based on the known rate of disintegration of radioactive phosphorus. The virus isolated was estimated to contain about 3 microcuries of radioactivity.

from the first 3 ultracentrifugations were combined and divided into protein and protein-free fractions by means of precipitation of protein with hot 5 per cent trichloroacetic acid. These latter fractions, together with the mash or press cake, the wash water from the sand and roots, and the supernatant liquid from the 4th ultracentrifugation, were each dried and incinerated. The 620 mg. of purified virus obtained from the plants fed radioactive phosphorus were prepared for analysis by simply freezing and drying. The various fractions were then examined for radioactivity by means of a Lauritsen type electroscope. The results which were obtained are summarized under Experiment 1 in Table I. It may be seen that 12.3 per cent of the total radioactivity which was recovered was associated with the virus. The fact that

the supernatant liquid from the 4th ultracentrifugation was found to be devoid of radioactivity, whereas radioactive phosphorus in the form of disodium phosphate may be separated readily from the virus, is good evidence that the radioactive phosphorus is integrally bound to the virus, presumably in the nucleic acid portion of the molecule.

A second experiment similar to the one just described was next carried out, except that a total of 450 mg. of disodium phosphate containing 11.4 millicuries of radioactivity was used and the feeding period was extended to 40 days, following which the plants were removed and frozen. The sets of normal plants fed ordinary and radioactive phosphorus weighed 403 gm. and 393 gm., respectively, whereas the corresponding sets of diseased plants weighed 222 gm. and 213 gm., respectively. The yield of purified virus from the 3 diseased plants fed radioactive phosphorus was 740 mg. and that from the plants fed ordinary phosphorus was 610 mg. In this experiment one-half of the purified virus containing radioactive phosphorus, together with the remaining fractions from both normal and diseased plants fed radioactive phosphorus, was incinerated. These preparations were then tested for radioactivity. In order to reduce inaccuracies in the tests due to self-absorption in the bulkier samples, all preparations were then taken into solution and the phosphorus was precipitated as phosphomolybdate. The phosphomolybdate precipitates were incinerated and tested for radioactivity. Following this treatment, the activities of the virus and of the two mash samples were approximately doubled, those of the protein components of the supernatant fluids were increased by about 20 per cent, and those of the remaining samples were essentially unchanged. The activities recorded in Table I for Experiment 2 are for the phosphomolybdate precipitates and probably provide a more accurate picture of the distribution of phosphorus than the values recorded for Experiment 1. It may be seen that 70 to 75 per cent of the radioactivity was found in the normal and diseased plants, and of that in the diseased plants about 30 per cent was in the virus. The supernatant fluid obtained on ultracentrifugation of the juice from normal plants contained more radioactivity than the corresponding fraction from the diseased plants. As in the previous experiment, the supernatant liquid from the 4th ultracentrifugation of the virus possessed no measurable amount of radioactivity. The experiments demonstrate that it is possible to secure purified tobacco mosaic virus containing considerable radioactivity.

In an effort to learn something of the distribution of radioactivity following inoculation of virus containing radioactive phosphorus, the lower leaves of 9 medium-sized normal Turkish tobacco plants were thoroughly rubbed by means of a bandage gauze pad with 15 ml. of a solution containing 58 mg. of the radioactive virus in 0.1 M phosphate buffer at pH 7. The plants were watered as usual and after 12 days the inoculated leaves were removed and



frozen. The upper uninoculated portions of the plants were also removed and frozen. The two portions were macerated and the virus was isolated from each by ultracentrifugation. The supernatant liquids obtained on ultracentrifugation as well as all insoluble material were added to the respective press cakes and these were incinerated, taken into solution, and the phosphorus precipitated as the phosphomolybdate. The 316 mg. of purified virus from the inoculated leaves, as well as the 204 mg. of virus from the uninoculated portions of the plants, were also incinerated and the phosphorus precipitated as the phosphomolybdate. Following ignition, the 4 samples were tested for radioactivity, and the results which were obtained are presented in Table II. It may be seen that most of the radioactivity was found to be associated with non-virus components, of which about 40 per cent was in the inoculated and about 60 per cent in the uninoculated portions of the plants. A small but

TABLE II

*Distribution of Radioactivity in Turkish Tobacco Plants 12 Days following Inoculation of Lower Leaves with 58 Mg. of Radioactive Tobacco Mosaic Virus\**

Virus isolated from inoculated leaves.....	8.3
All material of inoculated leaves except virus. ....	33.4
Virus isolated from upper uninoculated leaves.....	5.8
All material of uninoculated leaves except virus.....	52.5

\* The radioactivity was measured by means of a Geiger-Müller counter. The numbers represent the per cent of radioactivity found in the various fractions of the plants. The tests were made on the incinerated phosphomolybdate precipitates obtained from the various fractions. The radioactivity recovered represents 70 per cent of the 0.2 microcurie of radioactivity that was applied in the form of virus.

significant amount of radioactivity was found in virus isolated from the uninoculated portions of the plants. However, the fact that most of the radioactivity was found in the non-virus portion of the uninoculated parts of the plants and much radioactivity in the similar portions of the inoculated leaves proves that most of the radioactivity applied in the form of virus was converted into a non-virus form. Therefore, the isolation of radioactive virus from the uninoculated portions of the plants has no significance, for it could have been produced from radioactive phosphorus absorbed and transported in a non-virus form. Since it is likely that only a small portion of the virus in the inoculum was actually introduced within the cells as virus (7), it would follow that most of the radioactive phosphorus was actually absorbed later in the form of disintegration products of virus presumably formed on the surface of the leaves. It was determined that about 70 per cent of the radioactivity applied in the form of virus was actually isolated in the 4 fractions; hence, there was little loss due to washing of virus or of disintegration products from the leaves in the 12 day period during which the plants were watered.

It appeared desirable to determine the extent to which virus can be removed from inoculated leaves by means of vigorous washing immediately following inoculation. Accordingly, all of the leaves of 2 sets of 7 plants each of normal medium-sized Turkish tobacco plants were inoculated with two 16 ml. portions of 0.1 M phosphate buffer at pH 7 containing 4 mg. of ordinary tobacco mosaic virus per ml. The leaves of one set of plants were then removed and each leaf was well washed in a running stream of tap water. The leaves of the other set of plants were also removed and both sets of leaves were placed in a room held at  $-12^{\circ}$  within  $\frac{1}{2}$  hour of the start of the experiment. After standing for 2 days at  $-12^{\circ}$ , the frozen leaves were macerated and the virus was isolated by differential centrifugation. The actual yield of virus from the unwashed leaves was 45 mg. or 70 per cent of the virus applied to the leaves. The actual yield of virus from the washed leaves was 27 mg. or 42 per cent of that applied to the leaves. These results indicate, in view of the losses known to occur during the purification of such small amounts of virus, that practically all of the virus applied was retained either on or within the unwashed leaves and that well over 50 per cent of the virus applied was retained in the case of the washed leaves. The fact that it is difficult to differentiate between radioactive phosphorus accepted in the form of virus and that absorbed in the form of virus disintegration products and the lack of information concerning the extent of the multiplication of virus immediately following inoculation make it exceedingly hazardous to attempt to determine the manner of virus reproduction by means of virus labeled with radioactive phosphorus.

#### DISCUSSION

When mosaic-diseased Turkish tobacco plants were fed a nutrient solution containing radioactive phosphorus in the form of disodium phosphate over a period of several weeks, about 30 per cent of the radioactive phosphorus taken up by the plants was isolated in the form of purified tobacco mosaic virus. The fact that the radioactive phosphorus was not separated from the virus on ultracentrifugation is an indication that it was integrally bound, presumably in the nucleic acid component of the virus. This result is in accord with that described in a preliminary paper by Born and coworkers (9), who noted that ordinary tobacco mosaic virus absorbed only an insignificant amount of radioactivity when allowed to stand for several days in a solution of sodium phosphate containing radioactive phosphorus. In the present work the proportion of radioactive phosphorus associated with the virus was considerably greater than that described by Born and coworkers, who found about 87 per cent of the radioactivity in the press cake and sediment of chloroplasts and only about 2.1 per cent with the purified virus.

The preparation and isolation of tobacco mosaic virus possessing high radioactivity made it possible to determine the distribution of radioactivity follow-

ing the inoculation of such virus to normal Turkish tobacco plants. Unfortunately, in this experiment most of the radioactivity was found to be associated with non-virus components in both inoculated and uninoculated portions of the plants; hence, it was impossible to be certain that the small amount of radioactive virus found in the uninoculated portions of the plants arose as a result of actual movement of inoculated virus. If, as has been generally assumed, only a very small part of virus applied to the surface of a leaf actually enters the cells of the leaf in the form of active virus, it would follow that most of the radioactive phosphorus taken up by the plants in this experiment was absorbed in a non-virus form. It seems likely that it will be exceedingly difficult to distinguish between radioactive phosphorus taken up by the plant in the form of virus and that taken up in the form of virus disintegration products. The present work indicates that there are major difficulties involved in the use of tobacco mosaic virus containing radioactive phosphorus as a means for determining the nature of virus reproduction.

The experiments described were conducted with the assistance of Mr. Marshall Barbour.

#### SUMMARY

Normal and tobacco mosaic-diseased Turkish tobacco plants were grown in sand for a period of several weeks, during which they were fed daily a complete nutrient solution to which had been added disodium phosphate containing radioactive phosphorus. Determinations were made of the distribution of radioactive phosphorus in different fractions such as the wash from the sand and roots, the press cake obtained on pressing the juice from the plants, the protein and protein-free portions of the supernatant liquids obtained on ultracentrifugation of the juices, and the purified tobacco mosaic virus isolated from the diseased plants. Chemical analyses as well as radiographs of the normal and diseased leaves indicated that they contained the same amount of phosphorus. Approximately 30 per cent of the radioactive phosphorus absorbed by the diseased plants was found to be combined with the purified tobacco mosaic virus that was isolated from these plants. Following the inoculation of purified tobacco mosaic virus possessing high radioactivity to normal Turkish tobacco plants, most of the radioactivity was found to be associated with non-virus components of which about 40 per cent was in the inoculated and 60 per cent in the uninoculated portions of the plants. Although a small amount of radioactive virus was isolated from the uninoculated portions of the plants, it was impossible, because of a number of complicating factors which have been discussed, to draw from the results any reliable conclusions regarding the mode of reproduction of tobacco mosaic virus.

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## EXPLANATION OF PLATES

Photographed by J. A. Carlile

## PLATE 6

FIG. 1. Contact radiographs of leaves of normal (*a, c, e*) and tobacco mosaic-diseased (*b, d, f*) Turkish tobacco plants 1 (*a, b*), 2 (*c, d*), and 3 (*e, f*) weeks, respectively, after the start of a daily application of a nutrient solution containing radioactive phosphorus. Although the radioactive phosphorus appears to be well dispersed throughout the leaves, the lightest areas, which are indicative of the greatest concentration of radioactivity, conform to the major veins of the leaves. The diseased leaves are from plants described in Experiment 1, Table I.

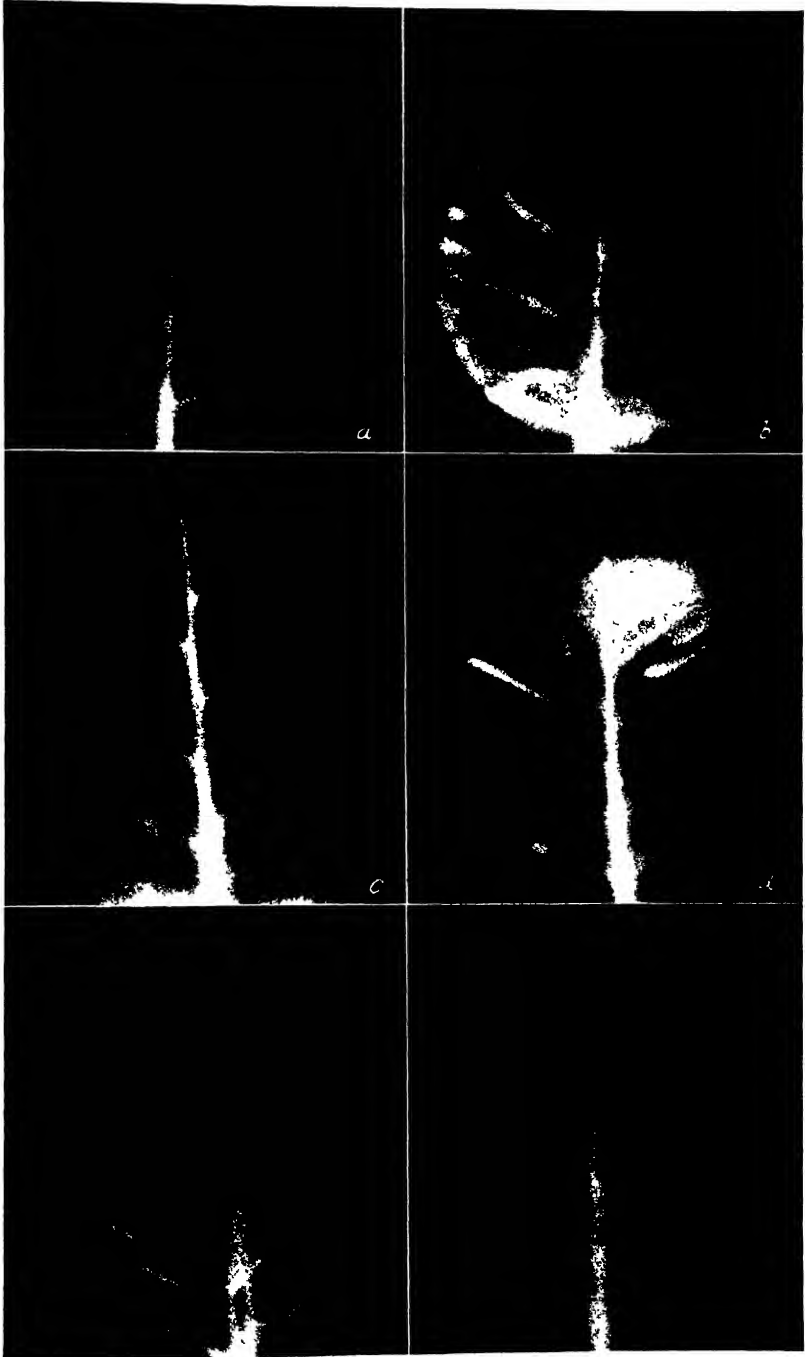
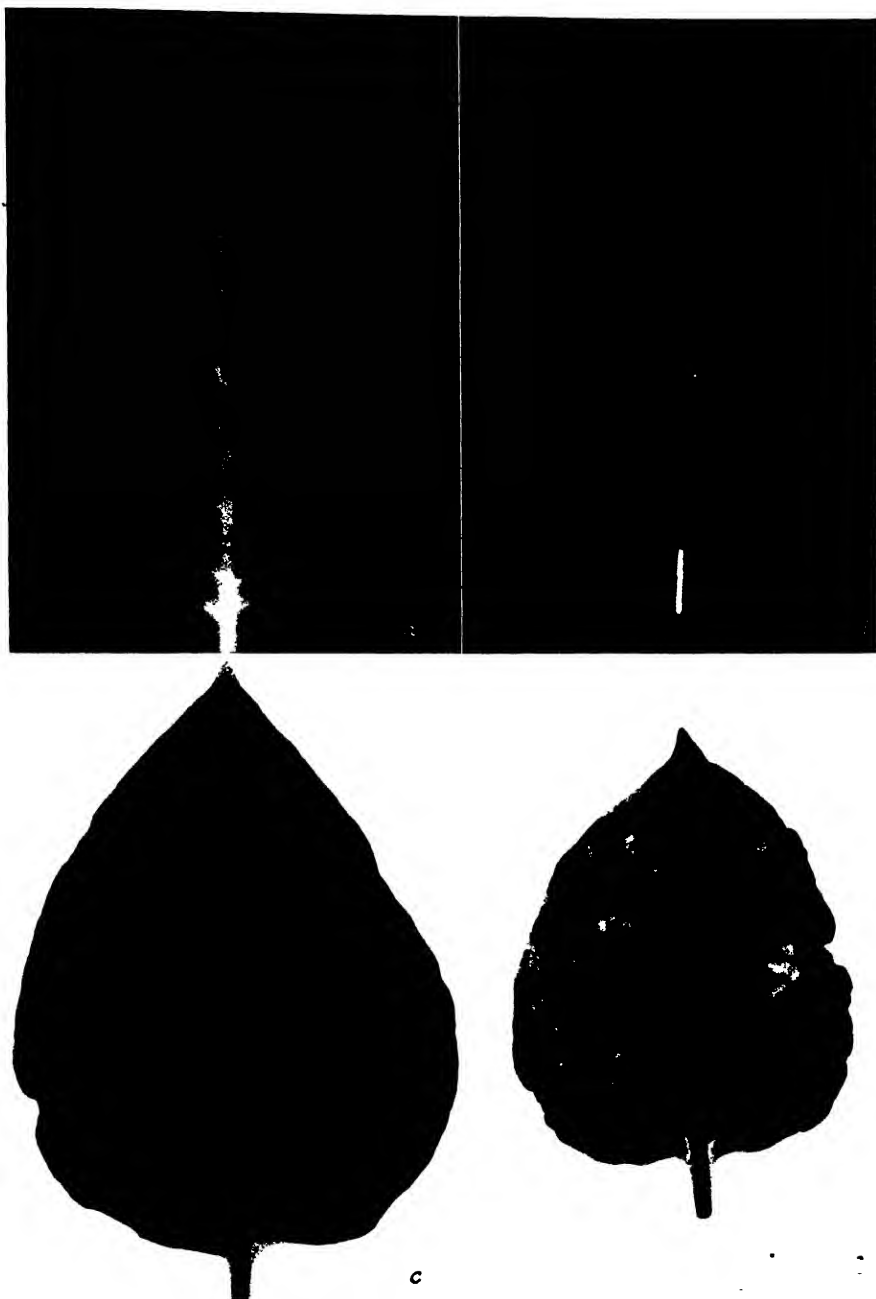


FIG. 1

PLATE 7

FIG. 2. Contact radiographs and photographs of leaves of normal (*a, c*) and tobacco mosaic-diseased (*b, d*) Turkish tobacco plant 7 weeks after the start of the experiment in which a nutrient solution containing radioactive phosphorus was fed for 30 days. The leaves are from the same plants used for Fig. 1.



C

FIG. 2

(Stanley: Radioactive tobacco mosaic virus)





## RESPIRATORY EFFECTS UPON THE VISUAL THRESHOLD

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In a completely dark adapted individual the absolute threshold of vision remains constant within narrow limits indefinitely. The photochemical systems of the receptors are at rest; and this state is not disturbed appreciably by the minimal exposures to light needed to determine the threshold. Under such circumstances various types of physiological stress cause marked changes in threshold which must originate at levels central to the photochemical system itself. In this sense the threshold of the dark adapted eye is an indicator of central nervous imbalance.

It has already been shown that lowering the oxygen tension of the inspired air to about 10 per cent, while it does not affect the course of visual adaptation, raises the threshold 2 to 4 times (Bunge, 1936; McDonald and Adler, 1939; McFarland and Forbes, 1940-1941). Lowering the blood sugar with insulin has been reported to induce a similar response (McFarland and Forbes, 1940-41).

The present paper is concerned with the effects upon the threshold of low oxygen tensions, applied gradually or suddenly, and of short and long duration; and of changes in the rate of breathing and in the composition of the inspired gases. All thresholds have been measured in the periphery of the dark adapted eye and involve the function of the rod apparatus alone.

### I

#### *Apparatus and Methods*

Visual thresholds were measured with an adaptometer which has already been described (Wald, Jeghers, and Arminio, 1938). In this instrument the intensity of the test field is regulated with a pair of circular neutral wedges, superimposed in reverse so as to compensate each other. The full white radiation from a tungsten filament lamp was employed.

The subject faced circular test fields which subtended visual angles of  $1^\circ$  or  $2^\circ$  and were centered respectively  $13^\circ$  or  $10^\circ$  below a red fixation star. A shutter controlled by the subject exposed the field for flashes 1/50 second in duration. The fixation point remained on at all times. All observations were monocular, usually on alternate eyes. During most experiments each subject wore a pair of spectacles in which the lenses had been replaced with thin brass discs pierced with circular

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\* This research has been supported in part by a grant to G. W. by the Josiah Macy, Jr., Foundation.

openings 1.6 or 2 mm. in diameter. These served as artificial pupils to guard against the possibility that any of the recorded changes in threshold were due to fluctuations in pupil area.

Gas mixtures were prepared in large cylinders, and for short term experiments were transferred to a Douglas bag. The subject wore a nose clip and breathed through a mouthpiece communicating with a two-way valve, so that only the mixtures could be inhaled and all expired gases escaped into the room.

## II

### *Low Oxygen Tensions*

Our first experiments on the effects of low oxygen tensions, performed in 1937, confirm in general the observations of Bunge, of McDonald and Adler, and of McFarland and Forbes. The discussion which follows is restricted primarily to aspects of the experiments which amplify and extend these previous reports.

In this series of experiments no attempt was made to regulate the rate or depth of breathing with the thought that in this way automatic compensatory mechanisms would be given free play to meet the anoxia. A 1° test field was used, centered 13° below the fixation point. Artificial pupils were worn in some of the experiments; beyond raising the general level of the threshold they did not appreciably modify the results.

Two types of experiment were performed, one in which the anoxia was of short duration and began and ended abruptly; and another conducted in a gas chamber in which the anoxia was established gradually and maintained for a number of hours.

In the first type of procedure completely dark adapted subjects, breathing through a mouth-piece, were switched instantaneously from room air to air-nitrogen mixtures containing 8 to 11 per cent oxygen. After 20 to 30 minutes the subject was returned abruptly to room air.

The simplest and most usual type of response to this procedure is shown in Fig. 1 A. In almost all cases the threshold rose within 1 to 10 minutes of anoxia to new levels 0.2 to 0.5 log unit above normal. In repeated experiments with 6 subjects this change averaged 0.35 log unit or a rise of about  $2\frac{1}{4}$  times. Occasionally after reaching the anoxic level the threshold fluctuated widely, rising for short intervals to as much as 0.7 to 1.0 log unit above normal. On returning to room air the threshold ordinarily fell within a few minutes to the normal level.

Certain subjects yielded results which departed from this pattern in characteristic ways. In Subject J. B. the threshold behaved in an orthodox way during the anoxia, but on the return to room air it fell rapidly to far below the normal level and remained subnormal for 10 to 15 minutes (Fig. 1 B). This subject also described vivid visual hallucinations during this period,

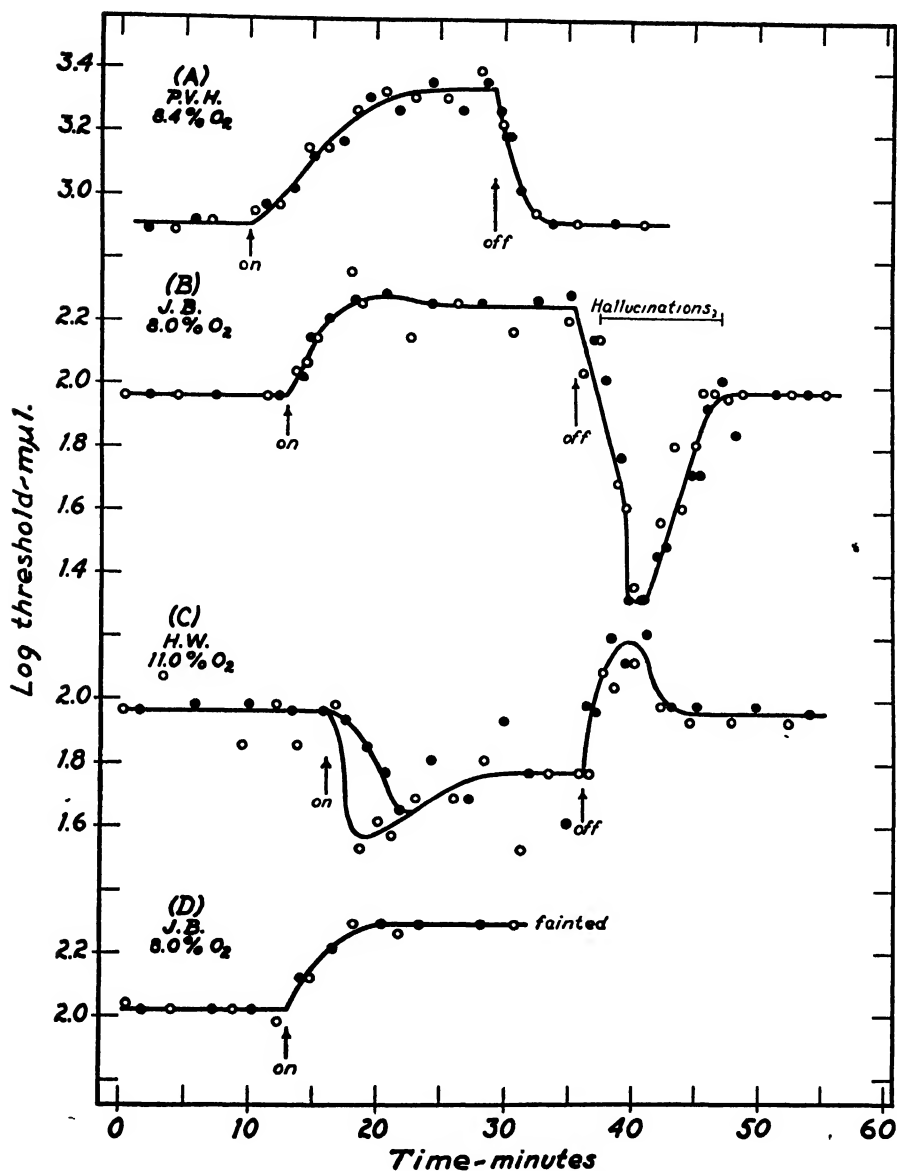


FIG. 1. Changes in the visual threshold on sudden exposure to and release from low oxygen tensions with breathing uncontrolled. Each point represents a single measurement of the threshold. In Experiment A artificial pupils were worn; B-D were performed with natural pupils. Open circles show measurements on the right eye, solid circles those on the left eye. Thresholds in millimicrolamberts ( $=10^{-6}$  millilamberts).

replacing one another rapidly and with no apparent continuity of subject, for as long as 12 to 15 minutes. Subnormal thresholds appeared in 4 and hallucinations in 6 out of 7 completed experiments with this subject. The hallucinations did not depend therefore on visual hypersensitivity; yet when both phenomena appeared together they coincided roughly in time.

Another subject yielded a pattern of threshold changes the mirror image of that just described (Fig. 1 C). In 2 out of 3 experiments his threshold *fell* during the anoxia. The records of these experiments contain the significant notation, "Puffs very hard; breathes gas up very rapidly." In all experiments with this subject the threshold rose sharply on the return to room air and remained for a time well above the normal level.

These wide variations in response may be ascribed primarily to characteristic differences in breathing pattern among the subjects. They did not appear in later experiments with 2 subjects in which the rate and depth of breathing were controlled. As shown below such supersensitivity as appears in Fig. 1 B and 1 C may be produced at will by hyperventilation; and the sequence of changes in Fig. 1 C was apparently due to the hyperventilation actually observed during the anoxia followed by a period of apnea on the return to room air.

The magnitude of the observed changes seems to represent the limit to which the visual threshold can be displaced by these means. It did not vary consistently with the oxygen tension between 8 and 11 per cent; nor was the rise in threshold obviously related to the distress experienced by the subjects. During anoxia subjects frequently reported sensations of dizziness, numbness, and cold in the extremities, feelings of restlessness, and flickering "subjective" light and color sensations, in no apparent relation with the behavior of the threshold. All subjects reported red "subjective" light starting about 15 to 30 seconds after the return to room air and lasting 2 to 3 minutes. One experiment ended when the subject fainted with his threshold established at a stable level 0.27 log unit above the norm (Fig. 1 D). It may be concluded from these observations that the visual threshold reflects only a restricted segment of the central nervous changes in anoxia.

Through the courtesy of Dr. D. B. Dill we were permitted to participate in anoxia experiments at the Fatigue Laboratory of Harvard University in 1937. These were conducted in a gas chamber in which the air was diluted with nitrogen without change in total barometric pressure. Oxygen contents of 11 to 12 per cent, simulating altitudes of 15,000 to 17,000 feet, were attained within 30 to 90 minutes after the first introduction of nitrogen, and were maintained thereafter for about 5 hours. Data from two such experiments, performed with natural pupils, are shown in Table I and in Figs. 2 and 3. A third experiment in which artificial pupils were worn yielded similar results.

These experiments indicate that the visual threshold rises with the first

dilution of oxygen (Fig. 2). The scatter of individual measurements is such that from 20.9 to about 14 per cent oxygen (10,000 to 11,000 feet) there is some overlap of thresholds with the norm; yet the average threshold rises slowly throughout this interval. As the oxygen is diluted below about 14 per cent the threshold increases rapidly, in the present subject to a level about 0.35 unit or  $2\frac{1}{2}$  times above normal, at about 11 per cent oxygen (17,000

TABLE I

Data of two experiments performed in a gas chamber in which the air was diluted with nitrogen at sea level atmospheric pressure. Subject P. V. H. Thresholds in millimicro-lamberts ( $10^{-6}$  millilamberts).

Oxygen	"Altitude"	Time	Average log threshold		No. of readings averaged	Remarks
			Right eye	Left eye		
<i>per cent</i>	<i>feet</i>	<i>min.</i>				
20.9	0	0	2.02	2.03	12	
18.1	4000	3-12	2.07	2.07	5	
16.8	6000	18-32	2.14	2.09	5	
16.1	7000	33-40	2.08	2.09	4	Uncomfortable; puffing
15.6	7800	42-53	2.08	2.10	4	
14.0	10500	56-67	2.11	2.13	4	
12.3	13800	76-96	2.18	2.16	6, 7	Dizzy; periodic breathing
11.8	15000	98-115	2.21	2.17	5	
11.3	16000	120-139	2.25	2.25	5	Sick; yawning
11.3	16000	147-177	2.29	2.27	7	Sick
11.3	16000	262-292	2.30	2.29	7	Sick
20.9	0	299-320	2.01	2.00	6, 5	
20.9	0	0	2.05	2.03	4	
10.9	17000	58-120	2.34	2.34	15, 16	
11.3	16000	240	—	—	—	Arterial saturation 74.5 per cent
10.9	17000	316-333	2.34	2.32	4	
10.9	17000	373-391	2.38	2.38	5	
20.9	0	398-405	2.06	2.07	3	

feet). The form of this relation suggests that with still further decrease in oxygen tension the threshold might rise much higher. Actually, however, this is close to the limiting tolerance of unacclimatized subjects. As Table I indicates the present subject experienced serious distress even at these levels and probably could not have continued the experiment at still lower oxygen pressures.

Some time after the anoxic threshold had been well established in each of these experiments the subject was highly light adapted and his complete dark adaptation curve was measured. All thresholds, rod and cone, were found to be about equally elevated on a logarithmic scale, *i.e.*, multiplied by an

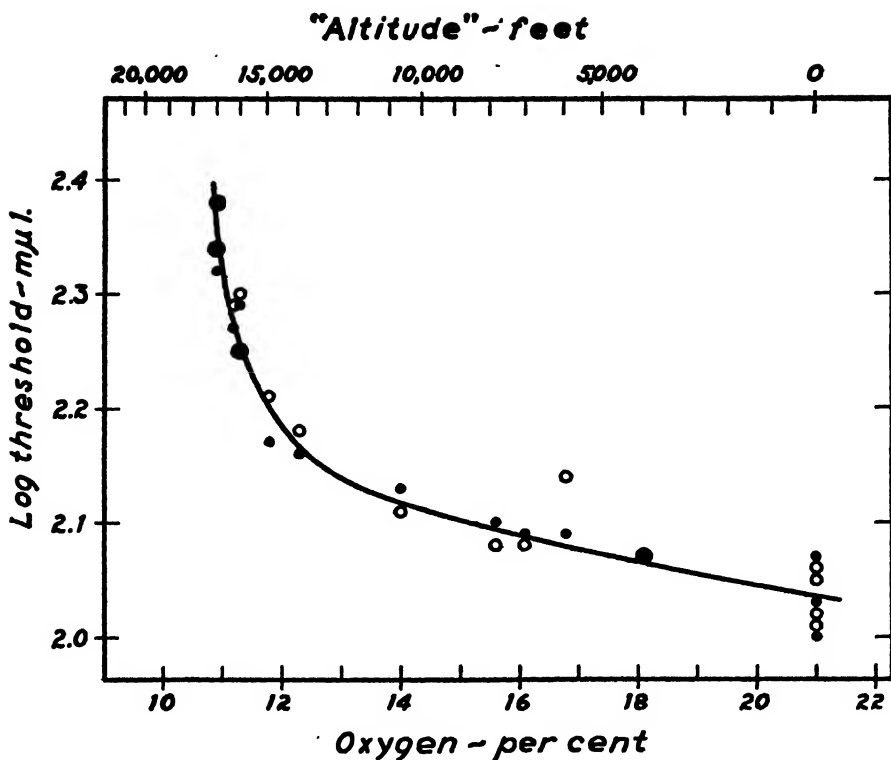


FIG. 2. The variation of visual threshold with decrease in oxygen tension or rise in simulated altitude. Data from Table I. Open circles, right eye; solid circles, left eye. Subject P. V. H.

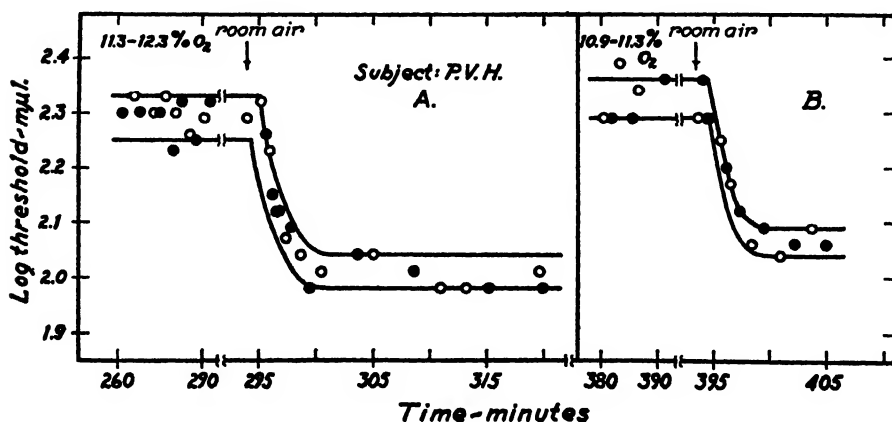


FIG. 3. Descent of the threshold to normal following 5 to 6.5 hours exposure to low oxygen tensions. Open circles, right eye; solid circles, left eye.

approximately constant factor. The speed of rod and cone dark adaptation appears to be entirely unaffected by anoxia (*cf.* Bunge; McDonald and Adler; McFarland and Forbes).

At the close of each experiment the subject was returned abruptly to sea level atmosphere. The details of the descent of his visual threshold are shown in Fig. 3. After exposures of 5 and 6.5 hours to diminished oxygen tensions the threshold returned to its normal level within 5 to 6 minutes, and without incident.

The changes recorded in these long term experiments agree in magnitude with those obtained with the same subject on sudden and short exposure to anoxia (Fig. 1A). The adjustment of central nervous structures to low oxygen tensions appears to be a rapid process. Anoxias of several hours duration cause changes in the threshold which neither exceed those recorded during the first few minutes nor persist longer after the return to room air.

### III

#### *Acid-Base Imbalance*

The following experiments are concerned with the effects of respiratory acid-base imbalance on the threshold. The subjects breathed to the beat of a metronome, so far as possible without change in amplitude; and the rate and depth of respiration were recorded by pneumograph. Thresholds were measured in a 2° field centered 10° below the fixation point, and artificial pupils 2 mm. in diameter were worn at all times. As in previous experiments the subjects were kept completely dark adapted throughout. The results of these experiments are shown in Fig. 4 (Subject H.C.G.) and Fig. 5 (Subject H.P.K.).

On increasing the rate of breathing room air by 50 to 100 per cent the threshold fell, usually within 5 to 10 minutes, to about half its normal value (Figs. 4 *a* and 5 *a*). In 22 experiments with 2 subjects this decrease ranged from 0.13 to 0.41 log unit and averaged 0.27 log unit or a decline of 47 per cent. On returning abruptly to the normal rate of breathing the threshold rose to normal usually within 2 to 3 minutes.<sup>1</sup>

Oxygen tensions of 32 to 36 per cent do not appreciably affect these results (Fig. 4 *b*; Fig. 5 *b*, *c*). The hypersensitivity induced by rapid breathing is not due therefore to increased oxygenation of the blood.

It is, however, due to the alkalosis associated with hyperventilation, and can be abolished or reversed by adding carbon dioxide to the inspired gases. The presence of 2 per cent carbon dioxide in mixtures containing 32 to 36 per cent oxygen does not affect the threshold when breathed at the normal

<sup>1</sup> Gellhorn (1936 *a*) has reported that rapid breathing *raises* the threshold for brightness discrimination. The relation of these to the present experiments is obscure.



rate, but completely abolishes the fall in threshold otherwise associated with rapid breathing (Fig. 4*c*; *d*; Fig. 5*d*). When the carbon dioxide content is raised to 5 per cent the threshold rises 0.2 to 0.5 log unit at both normal and rapid rates of ventilation (Fig. 4*e*, *f*; Fig. 5*e*, *f*).

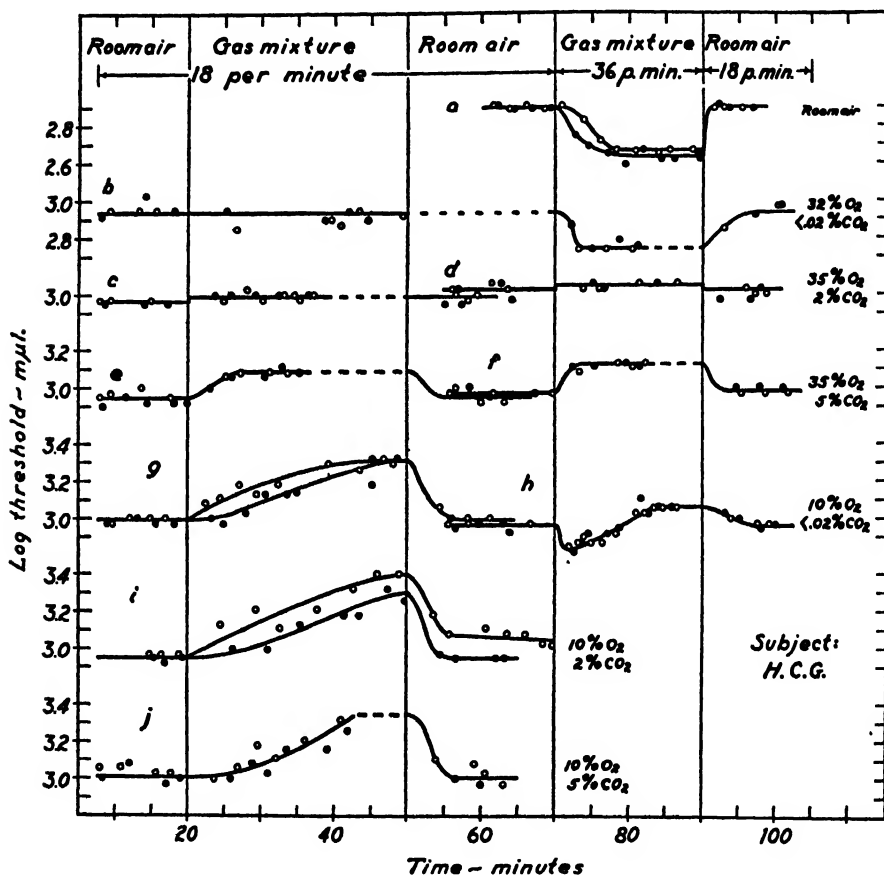


FIG. 4. Effects on the visual threshold of variations in the rate of breathing and in the composition of the inspired gases. Rates of breathing are shown at the top of the figure, gas composition to the right of each experiment. In this subject the normal uncontrolled rate of breathing in room air was 18 per minute. Open circles, right eye; solid circles left eye.

Respiratory alkalosis and acidosis therefore exert opposed effects upon the visual threshold. The former depresses, the latter raises the threshold in about equivalent degree, averaging in the present experiments about 0.3 log unit or a factor of about 2.

## IV

*Anoxia and Acid-Base Imbalance*

These experiments are concerned with the interaction of acid-base imbalance and low oxygen tensions upon the visual threshold. The rate and depth of breathing were controlled and artificial pupils were worn at all times.

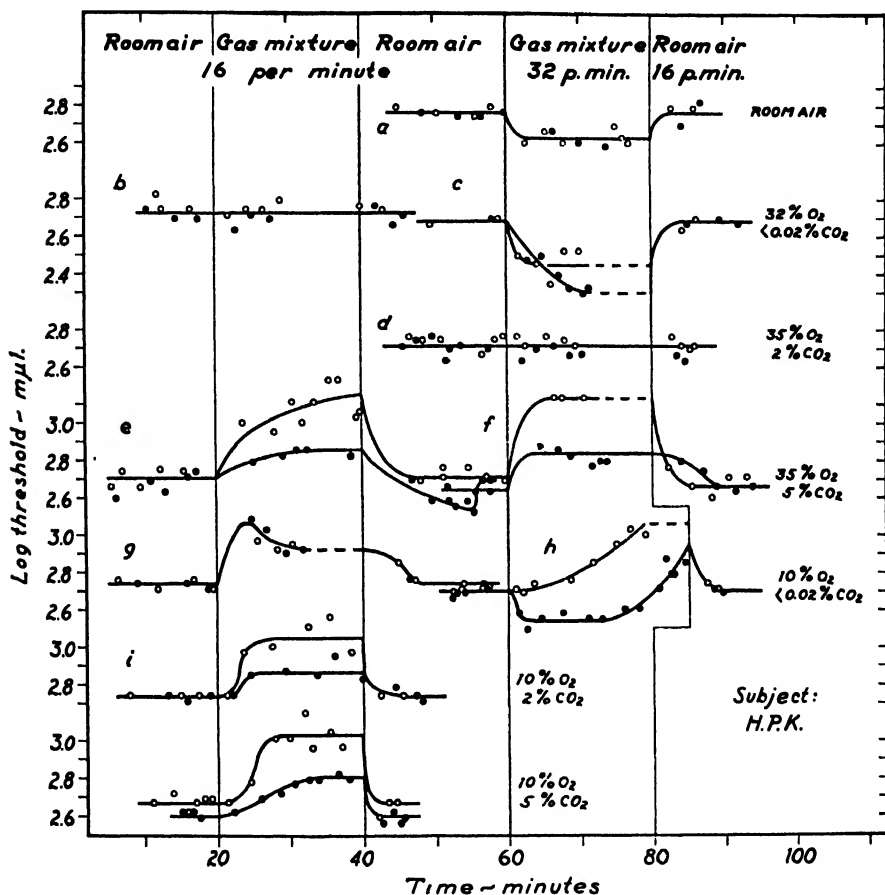


FIG. 5. Effects on the visual threshold of variations in respiratory rate and in the composition of the inspired gases. In this subject the uncontrolled rate of breathing in room air was 16 per minute. Symbols as in Fig. 4.

The reaction to 10 per cent oxygen breathed at the normal rate (16 to 18 per minute) was essentially like that shown in Fig. 1 A in which the breathing had been uncontrolled, but was obtained invariably in the present experiments (Figs. 4 g and 5 g). The threshold rose in anoxia and fell promptly to normal

at its close with no appearance of the individual peculiarities and incidents which marked the earlier experiments.

A particularly interesting situation results when low oxygen is breathed at twice the normal rate (Figs. 4 *h* and 5 *h*). Usually the threshold falls at first; always it at least fails to rise for several minutes. Finally it always does rise, eventually to values above the normal level; though it may remain, as in Fig. 4 *h*, well below the level attained at lower rates of breathing. The effects of rapid breathing therefore usually at first predominate over those of simple anoxia. After a time some of this advantage is lost. It is very likely that further manipulation of the breathing rate might maintain a much more effective compensation of anoxia than is evident in the present procedure.

Breathing rapidly apparently also ameliorates the subjective symptoms of anoxia. When 10 per cent oxygen was breathed at the normal rate the subjects invariably reported such symptoms—"subjective" light, difficulty in concentrating, lassitude, and so on. Rapid breathing of the same mixtures elicited no comments whatever, except in one instance "feels O.K."

The addition of 2 per cent carbon dioxide to mixtures containing 10 per cent oxygen, when breathed at the normal rate, contributed no notable differences in behavior (Figs. 4 *i* and 5 *i*). Mixtures containing 5 per cent carbon dioxide and 10 per cent oxygen also produced no extraordinary changes in the threshold beyond those occasioned by low oxygen alone (Figs. 4 *j* and 5 *j*); but with these mixtures the subject usually was unable to regulate his respiration effectively. The pneumograph records showed large increases in the depth of breathing in almost all cases; and frequently the subject was unable also to keep the rate of breathing down to that set by the metronome. Panting, sweating, and general distress usually cut these experiments short. The subjects were unable to complete experiments in which either 2 or 5 per cent carbon dioxide in 10 per cent oxygen was breathed rapidly.<sup>2</sup>

## V

### DISCUSSION

The changes in visual threshold observed in the present experiments are due to changes in structures central to the photochemical system of the rods. It is not possible to locate them at present with greater precision. They may originate at any loci along the visual pathways from rod outer limbs to cortex. It is known that nerve cell bodies and synapses in contrast with axones are highly sensitive to oxygen want. Recently it has been shown also that in the

<sup>2</sup> These procedures should not be confused with experiments in which CO<sub>2</sub> is added to the inspired gases without control of breathing (*cf.* Gellhorn, 1936 *b*). In the latter case 2 to 3 per cent CO<sub>2</sub> may aid in compensating the anoxia by stimulating the respiration.

cat hyperventilation increases the pH of the cerebral cortex and simultaneously lowers its threshold for neural or electrical excitation; hypoventilation or any other means of decreasing the cortical pH raises the threshold (Dusser de Barenne, McCulloch, and Nims, 1937). Lehmann (1937) has made comparable observations on peripheral nerve; in the phrenic nerve of the cat the threshold for induction shocks falls linearly as the pH rises from 7.2 to 8.1.

It is beyond the scope of the present paper to discuss extensively the blood and tissue changes which accompany anoxia and changes in respiratory rate. It should at least be noted, however, that these stresses do not act independently, but are involved in a complex pattern of interrelationships. Anoxia usually stimulates rapid breathing, in part due to the acidosis which results from incomplete oxidation of metabolic products. Hyperventilation in turn tends to compensate initially for anoxia; by raising alveolar oxygen and lowering alveolar carbon dioxide tensions it promotes the oxygenation of hemoglobin in the lungs. Hyperventilation, however, also induces alkalosis which may intensify the anoxia in either of two ways: by inhibiting the discharge of oxygen from blood to tissues, and by provoking a period of apnea.

In our initial experiments all these potentialities were permitted free play and resulted in a great variety of reaction patterns (Fig. 1). On exposure to anoxia the threshold rose or fell; on return to room air it rose, or fell to normal or subnormal levels. Simple regulation of the rate and depth of breathing in later experiments brought all these phenomena under control. But it may be assumed that the latter experiments no less than their forerunners involved the continuous interaction of internal changes in both oxygen and hydron concentration.

These experiments raise a possibility which should be explored further. Since the threshold may vary in either direction in anoxia depending upon the breathing pattern, it should be possible to regulate the latter so as to obtain virtually no change in threshold, and hence an optimally compensated response. It is clear from our observations that subjects left to their own devices do not automatically establish optimal behavior.

In general these experiments support the thesis that the visual threshold offers a practicable quantitative index of physiological imbalance. It is more sensitive and reliable for this purpose than subjective awareness of distress or sensory change; and has the advantage over certain other types of physiological measurement that it provides an index of net change in the central nervous system itself, usually the first to fail in physiological stress (Barcroft, 1934).

This is probably the principal importance of such measurements. During light and dark adaptation the visual threshold varies through a range as great as 1:1,000,000. In contrast the variations induced by even extreme central nervous imbalance are relatively small. In the present experiments

they average about 1:2. In subjects exposed to about 10 per cent oxygen Bunge found the average rise of threshold to be about 3; McFarland and Forbes about 4; and McDonald and Adler (1939) about 2.5 times. These seem to represent the extreme limits of variation which disturbances central to the photochemical system can contribute to the visual threshold.

#### SUMMARY

Measurements are reported of the effects of respiratory stresses upon the absolute threshold of peripheral (rod) vision. Since subjects were kept wholly dark adapted and the photochemical system of the rods therefore stationary, the changes recorded may be assumed to have originated more centrally. To this degree the measurements provide a quantitative index of central nervous imbalance.

Breathing room air or 32 to 36 per cent oxygen at about double the normal rate causes the visual threshold to fall to approximately half the normal value within 5 to 10 minutes.

This change is due primarily to alkalosis induced by the hyperventilation, and can be abolished or reversed by adding carbon dioxide to the inspired mixtures. Normal or rapid breathing of 2 per cent carbon dioxide causes no change in threshold; with 5 per cent carbon dioxide the threshold is approximately doubled.

Breathing 10 per cent oxygen at the normal rate also approximately doubles the threshold. This effect is compensated in part by rapid breathing. When 10 per cent oxygen is breathed at twice the normal rate the threshold usually falls at first, then slowly rises to supernormal levels.

Due primarily to variations in their breathing patterns subjects yield characteristically different responses on sudden exposure to low oxygen tensions with breathing uncontrolled. The threshold may either rise or fall; and on release from anoxia it may rise, or fall to normal or subnormal levels. The threshold adjusts to anoxia rapidly; exposures lasting 5 to 6 hours do not produce greater or more persistent changes than those of much shorter duration.

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# THE RELATIONS OF TEMPERATURE TO THE POTASSIUM EFFECT AND THE BIOELECTRIC POTENTIAL OF VALONIA\*†

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The influence of temperature upon bioelectric potential has been occasionally investigated, both to initiate action potentials,<sup>1,2</sup> and to indicate the nature of the controlling mechanisms,<sup>3-5</sup> e.g. whether simple diffusion potentials are adequate to account for the effects, or whether metabolic, surface active, or other phenomena are also involved. Before conclusions can be drawn from the temperature data alone, it is desirable to combine their study with those of other conditions or agents known to be electrically effective. In *Valonia* the two most studied of these are the seawater (NaCl) dilution effect<sup>6</sup> and the potassium concentration effect.<sup>7</sup> In the earlier work on these, temperature was not experimentally varied, the measurements being made over a narrow seasonal range (16–23°). Wider alterations are here described, especially upon the potassium effect. (The NaCl dilution effect is much less sensitive to temperature.)

The methods are essentially those of previous studies,<sup>5-8</sup> the cells being supported on glass rings, and impaled from above with fine glass capillaries, sap-filled and leading to calomel electrodes via KCl-agar salt bridges. Cells were employed only after good healing of the impalement wound had occurred—from several days up to several weeks after impalement. For the normal sea water effects it was found necessary to renew the sea water frequently, since small amounts of KCl diffusing from the

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† Briefly reported as an abstract in Blinks, L. R., *Biol. Bull.*, 1940, **79**, 350.

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<sup>2</sup> Hill, S. E., *J. Gen. Physiol.*, 1934–35, **18**, 357.

<sup>3</sup> For a discussion of the earlier work, see Bělehrádek, J., *Temperature and living matter*, Berlin, Borntraeger, 1935, 113–4, 170, 186–8, 223, 226.

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<sup>5</sup> Blinks, L. R., and Darsie, M. L., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1937, **35**, 522.

<sup>6</sup> Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 445.

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<sup>8</sup> Blinks, L. R., *J. Gen. Physiol.*, 1930–31, **14**, 139.



external salt bridge can markedly influence the magnitude of the temperature effects, as shown below. The measurements were mostly made by an automatic recording potentiometer (Micromax) compensating every 3 seconds. Current flow was therefore insignificant, and no appreciably different results were obtained when a vacuum tube interstage was used to render the instrument electrostatic. The time curves given are usually direct tracings of such Micromax records. In most cases the solutions were gently stirred with a fine jet of air, which also speeded temperature equilibration. When quick changes of temperature were desired, vials containing the sea water or other solutions were brought in advance to the desired temperature, and rapidly substituted for the previous vial. In all cases the experimental vials were immersed in larger vessels, either thermostated or manually adjusted to within  $1/2^\circ$  of the indicated temperature during the exposure. A small thermometer projected directly into the vial, immediately next the cell. All measurements here reported were performed in a darkened room, with generally less than 1 foot-candle of light in the vicinity of the cell. The vessels were also usually jacketed with black paper.

Most of the measurements were performed during the winter months in Florida, where the two species *V. ventricosa* and *V. macrophysa* yielded essentially similar results. *V. ventricosa* displays on the whole a larger potassium effect which correlates with both its larger normal P.D. in sea water<sup>8</sup> and its greater change with temperature. Some of the earlier exploratory experiments were performed with *V. macrophysa* in Bermuda, and with cells brought from there, with concordant results.

### *Effects of Temperature upon the Normal Potential*

Fig. 1 shows some of the characteristic changes of P.D. of a cell immersed in sea water. On cooling the cell from  $23^\circ$  to  $13^\circ$  there is first a slight depression of P.D., but this soon recovers and increases well above the warmer value. On warming to  $25^\circ$  there is first an increase, but a later fall; cooling to  $15^\circ$  repeats the rise at  $13^\circ$ , to even higher values. And again a warming to  $25^\circ$  causes an initial slight increase, but a pronounced later fall, which only slowly recovers, not however, to the  $15^\circ$  value. Still greater warming, to  $35^\circ$ , produces not only a large initial cusp, but an appreciably higher final level, sometimes, though not always, as high as at  $15^\circ$ . The exact time curve of these effects varies somewhat, as seen in the rest of Fig. 1, but the main points remain: essentially, the P.D. is lower in the range around  $25^\circ$  than it is either at lower or higher temperatures. The same overall effect is likewise seen in Fig. 2, where instead of quick  $10^\circ$  changes, the cells were slowly warmed from  $10^\circ$  up to  $36^\circ$  in the course of about 2 hours. Here the P.D. rose to higher values at the warm end than at the cool, in cell B; but cell A, when cooled finally to  $8^\circ$ , showed at least as high a P.D. as at  $36^\circ$ , after an initial drop.

The difference in behavior of these two cells is characteristic at the lower temperatures. Thus while the P.D. is high in the range  $10$ – $15^\circ$ , it often tends to fall off sharply below about  $8^\circ$ ; when it does, the P.D. often does not recover rapidly on warming. This state also coincides with a greatly decreased or

negligible potassium effect (contrary to the response at 10–15°, as seen below). There is quite possibly a change in physical state at the region 4–8°—perhaps a solidification or rupture of the protoplasmic surface—which is not readily reversible. (There is also a drop in P.D. following exposure to temperatures over 40° C.) Fig. 3 shows a similar experiment, plotted against temperature instead of time. The saddle-shaped curve is hardly compatible

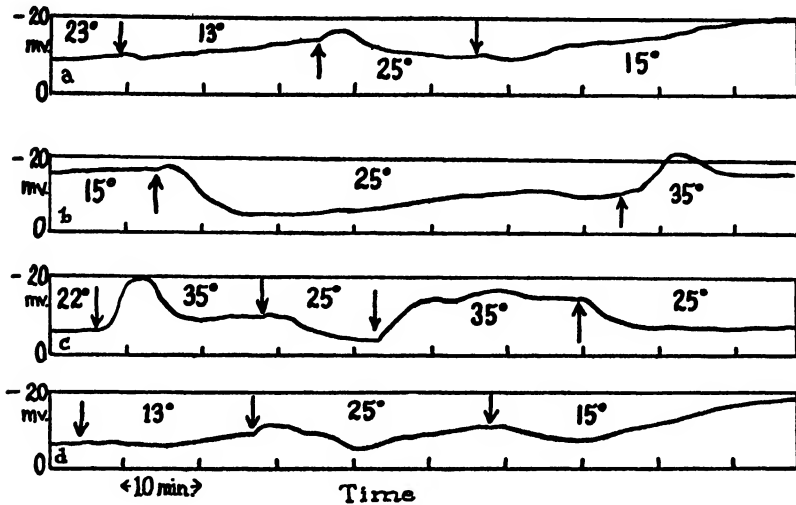


FIG. 1. Time course of P.D. change of impaled *Valonia ventricosa* in sea water. The temperatures were as indicated, changes being made at the arrows by substituting new vials already brought to the indicated temperature and maintained by water jacket. On changing from 23° to 13° there is a slight fall, followed by a rise; warming to 25° produces a rise then a fall, cooling to 15° a rise. Warming to 35° produces a sharp increase above 25° however, either as a cusp, or a maintained plateau. The curves are actual tracings of a continuous recording potentiometer record (Micromax). Time intervals of 10 minutes shown by vertical marks at bottom of records. Ordinates are potential difference across the protoplasm in millivolts, the sign being that of the exterior (here negative).

with either a thermodynamic potential (as at an electrode) or with a process closely related to metabolism. (The respiration of *Valonia* has been investigated over this temperature range<sup>9</sup> and shows a regular increase from low to high temperature, with a  $Q_{10}$  of around 2.) It is therefore desirable to inquire whether any other phenomena more closely parallel these P.D. changes, and if so, whether these may be responsible for the anomalous temperature course of the latter. Such parallel and control appear to be at hand in the potassium ion effects, next taken up.

<sup>9</sup> Unpublished experiments.

*The Temperature Effect at Various Potassium Concentrations*

The first suggestion that potassium might be implicated in the temperature effect came from the observation that occasional cells displayed larger changes than those shown in Figs. 1, 2, and 3. This often occurred with cells which were exposed after impalement to sea water from a dish where they had previously been kept for some time (to avoid osmotic or other changes at the

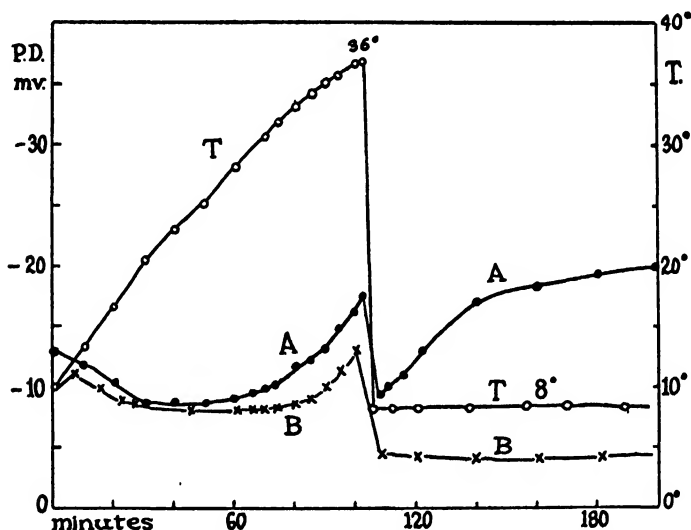


FIG. 2. Values of P.D. reached with two cells of *V. macrophysa*, first warmed steadily from 10° to 36° C., then cooled to 8°, as shown on the temperature curve "T." The P.D. of cells A and B both fell on warming to about 15° and remained about constant to 25°, above which it again increased. The P.D. of cell A first fell on sudden cooling to 8°, but later rose, while that of cell B remained low. Values derived from continuous Micromax recordings, switched alternately from cell A to B. P.D. values in millivolts at the left for curves A and B; temperatures at right for curve T. Time in minutes.

time of impalement and healing). Such water often had an increased KCl content, due to release of KCl from the vacuoles of other cells which had died in the vessel.<sup>10</sup> Enhanced temperature effects also occurred with cells when salt bridges had been left in the sea water for some time, permitting slow diffusion of KCl from the agar (see paragraph on methods, above). On renewing the sea water in both these cases, the temperature effect usually decreased

<sup>10</sup> Such increased KCl concentration is not injurious to the remaining cells: indeed it seems to aid their survival, probably by supplying sufficient potassium for normal accumulation, to keep pace with growth or slight evaporation.

markedly, although sometimes with characteristic changes due to removal of higher potassium concentrations (notably a temporary reversal of P.D. to a large positive value<sup>7</sup>).

These observations led to controlled experimental changes of the KCl content of the sea water, with the results shown in Fig. 4. When the normal sea water value of 0.012 M KCl was halved (by adding an equal volume of

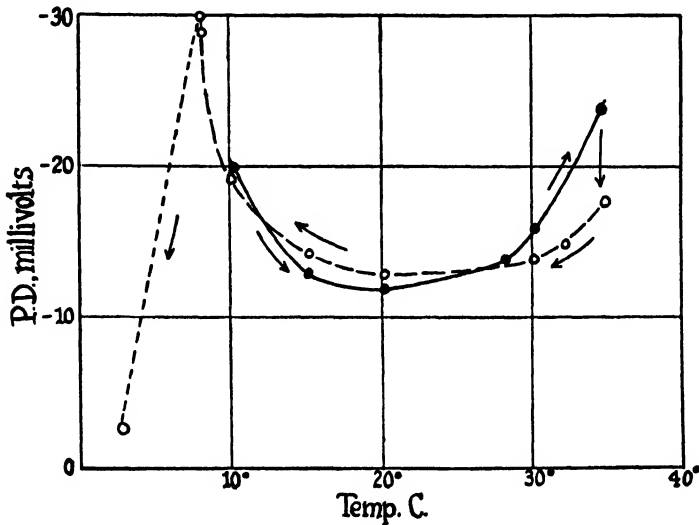


FIG. 3. Values of P.D. reached in another temperature run much like Fig. 2, here plotted against the temperature. A saddle-shaped curve, with a minimum between 15° and 25°, and rising both toward 10° and 35°, results. The drop at the lower end, at 4°, is not readily reversible, and is shown by a dotted line. The solid curve represents the steady values reached on an increasing, and then a decreasing temperature run, as indicated by the arrows. The higher value at 35° represents the initial, the lower a later steady value (*cf.* Fig. 1). Values taken from continuous Micromax record.

van't Hoff artificial sea water made up without KCl), the temperature effect was greatly diminished (Fig. 4 *a*). It was practically abolished in potassium-free sea water, but there are complications here due to the tendency for reversal of P.D.; in such a positive state the P.D. is very sensitive to a variety of agents, including temperature, but its time course is extremely unpredictable.

Increase of KCl concentration, on the other hand, back to 0.012 M restores the normal temperature effect, while doubling the sea water value, to 0.024 M KCl, enhances the response, which becomes greatly exaggerated at 0.1 M KCl (Fig. 4 *c*). Concentrations higher than this give not only a complex time course themselves<sup>7</sup> but also induce an irregular temperature response,

with exaggeration of the initial cusps, but no great alteration of permanent level. This is consistent with an "alterative" effect of these higher concentrations, found by Damon,<sup>7</sup> and probably occurring even at lower concentrations, though with subsequent recovery. (See Discussion.)

$\text{NH}_4\text{Cl}$  is even more effective in these respects than is  $\text{KCl}$ ; sufficient ammonia can enter the sea water from tobacco smoke in the room to increase the effects of temperature (as well as of light<sup>9</sup>). Dilution of the  $\text{NaCl}$  and other

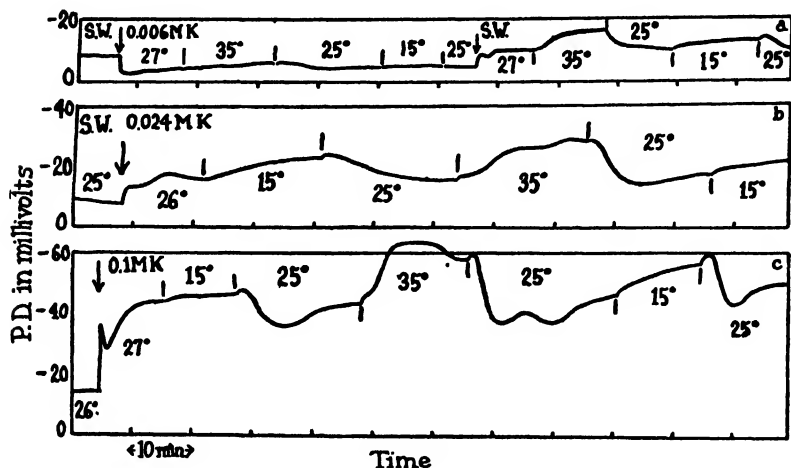


FIG. 4. The influence of  $\text{KCl}$  concentration on the temperature effect. The cell of *V. ventricosa* was changed from sea water (containing  $0.012 \text{ M KCl}$ ) to half this value,  $0.006 \text{ M KCl}$ , at the first arrow. Very little change of P.D. thereafter occurred either on warming to  $35^\circ$  or cooling to  $15^\circ$ . On restoration to normal sea water (S.W.) the normal temperature effect is regained (cf. Fig. 1); and on increasing the  $\text{KCl}$  content to  $0.024 \text{ M}$  and  $0.1 \text{ M}$  respectively in the next two panels, the temperature effect is correspondingly increased. Tracings of continuous Micromax records, the time marks being 10 minutes apart.

salts of the sea water (by isotonic glycerol) does not, however, greatly alter the temperature effect, provided the  $\text{KCl}$  concentration is kept constant by proper additions.

#### *Influence of Temperature on the Potassium Effect*

It seems hardly possible that  $\text{KCl}$  alters in strictly proportional fashion some other process controlling the P.D. so that it still seems best to ascribe its effects to its high diffusion potential in the protoplasmic surface, as was assumed by Damon and by Osterhout. The time curves show characteristically an increased negativity when  $\text{KCl}$  concentrations are increased, due to the high relative mobility of  $\text{K}^+$ , compared to the  $\text{Cl}^-$  (and of course to

Na<sup>+</sup>). For the higher KCl concentrations, this ordinarily takes the form of an initial sharp cusp, followed by a depression, and a later rise of negativity. Damon has interpreted such curves as due to an advancing wave of KCl which

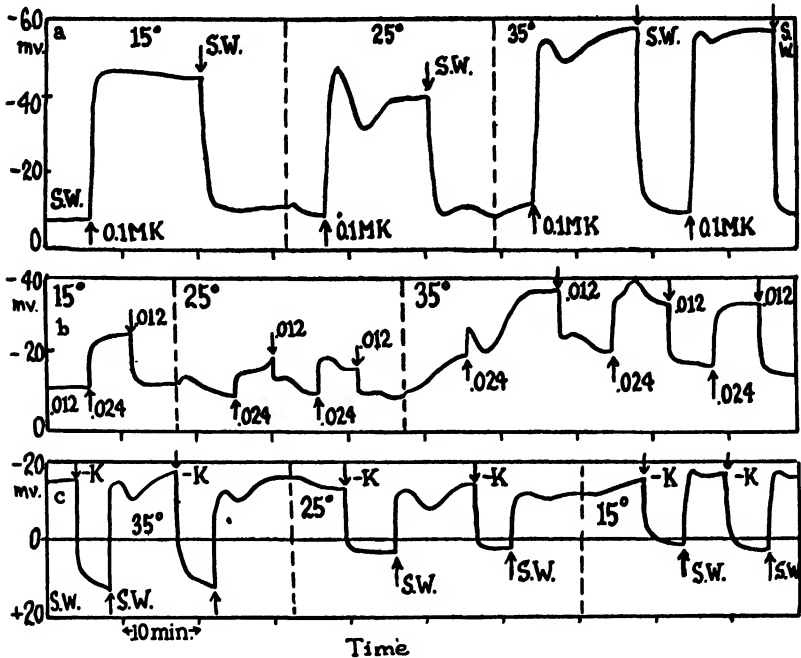


FIG. 5. Influence of temperature upon the magnitude and time course of the potassium effect in *V. ventricosa*. In (a) the arrow indicates the substitution of sea water containing 0.1 M KCl for normal sea water at the three temperatures 15°, 25°, and 35° C., with later return to sea water (S.W.). The p.d. is driven some 40 mv. more negative at 15° with a smooth-topped, regular time course; at 25° there is an initial cusp of about this size, but an abrupt fall, with later slow recovery. At 35° the cusp is considerably larger, the fall less, and the recovery faster. Essentially similar time courses at the three temperatures are found (b) for the doubling of KCl from 0.012 M in sea water to 0.024 M; also for substitution of potassium-free sea water ("—K"), and the return to normal sea water (c). P.D. across the protoplasm in millivolts; time intervals 10 minutes apart.

first establishes a diffusion potential across the outer surface (X), later abolishes this as its concentration builds up inside that surface, and finally produces a new negativity as it arrives at the inner surface (Y). These curves are duplicated in *V. ventricosa*<sup>8</sup> at the normal laboratory temperatures, being most cusped at 0.1 M KCl or higher, but showing at least rudimentary cusps with a slight fall and later rise even when the sea water value was doubled from

0.012 M to 0.024 M KCl (Fig. 5). This is generally true at 25°, although sometimes repeated later exposures give a flatter topped curve than the first, as seen in several cases in Figs. 5 and 6. A definite control is exercised by temperature upon the shape and magnitude of these time courses. Cooling to 15° generally gives perfectly regular, smooth curves, soon reaching a constant value, with scarcely an indication of cusp or later rise, even at 0.1 M KCl. This constant value may be 50 to 100 per cent higher than the average in-

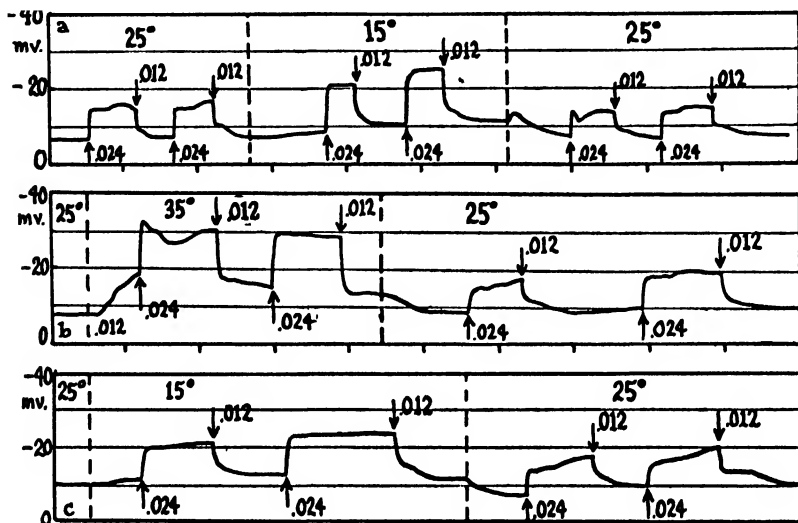


FIG. 6. Time courses of P.D. change when the sea water potassium level (0.012 M KCl) is doubled to 0.024 M, at the three temperatures 15°, 25°, and 35° C. The flat-topped response of some 14 mv. at 15° gives way to a smaller, slightly cusped response at 25°, and to a larger, often cusped response at 35°. P.D. across the protoplasm in millivolts; time intervals, 10 minutes apart. Changes of temperature indicated by the vertical dotted lines, of solutions by the arrows.

crease at 25°. However, at 35°, coinciding with the increased P.D. in sea water, the potassium effect is again increased, even over that at 15°. The time course is usually cusped, but not as markedly as at 25°, both the initial and final values being much higher, with a smaller depression between. (Repeated exposures may entirely smooth out these curves also, but their magnitude remains much greater than at 25°, as seen in several instances in Figs. 5 and 6.)

To a considerable degree, the same differences obtain when the potassium concentration is decreased, as for example when potassium-free sea water is substituted for normal sea water, as in Fig. 5 c. The P.D. change is greater

both at 35° and at 15° than at 25°, and the magnitude of the change closely parallels the P.D. value in sea water. (The tendency to reversal of P.D. at 35° is evident in Fig. 5 c, with potassium-free sea water.)

#### DISCUSSION

The close parallel at the different temperatures studied between the magnitude of the normal P.D., and of its increase by KCl, suggests that the two may have the same basis. Indeed, the practical abolition of both the P.D. itself, and of any temperature effect upon it at very low potassium concentrations, raises the question whether any other potential except that due to the external KCl concentration contributes toward the total P.D. across the protoplasm of *Valonia*. It is, of course, possible that the high KCl content of the sap sets up a large potential across the vacuolar surface of the protoplasm, which is in turn closely balanced by some other potential (perhaps due to organic ions of the protoplasm<sup>11</sup>). It has not yet proved possible to perfuse the vacuole of *Valonia* with new solutions, such as sea water, to determine the response of the vacuolar surface to potassium salts, but such experiments with *Halicystis*<sup>12</sup> suggest that the vacuolar surface is practically indifferent to them. If so, it is perhaps not surprising that the P.D. of *Valonia* is very slightly influenced by temperature when there is only 0.006 M KCl in the sea water—there may then be no other potential present to be altered. (Possibly two balanced potentials are being altered by temperature in exactly equal and opposite degree, but if so, this would still leave the external potassium concentration effect as the experimentally controlling factor.)

The simplest hypothesis is that a change in the mobilities of the ions  $K^+$  and  $Cl^-$  occurs in the protoplasmic surface, over the temperature range studied. The values of the *initial* cusps suggest that this does indeed change somewhat. But mostly it is the succeeding time course that differs; and here we may have several possible hypotheses to choose between:

(a) We may use the simple picture advanced by Damon<sup>7</sup> to account for the cusped time courses, namely an advancing concentration wave of KCl. Thus we may say that at 15° KCl enters very slowly, so that the original gradient is well maintained across the outer surface (*X*), hence the potential is steady. At 25° sufficient KCl enters to decrease the gradient across *X* fairly soon, hence the cusp, but the wave reaches the inner surface (*Y*) only slowly, hence sets up a second potential there only slowly and incompletely. At 35°, however, although there is rapid entrance across *X*, the wave also reaches *Y* very soon, and reestablishes a potential there. This attractive picture suffers

<sup>11</sup> Blinks, L. R., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, 8, 204.

<sup>12</sup> Blinks, L. R., *J. Gen. Physiol.*, 1934-35, 18, 409.



from the uncertainty as to whether there actually is any potassium effect at  $Y$ , as discussed above.

(b) We may invoke instead, "alterations" in the outer protoplasmic surface, induced by the potassium itself. There is considerable evidence<sup>9</sup> pointing to this as the cause of the depression following the initial cusp, for if one changes solutions, either back to sea water or up to higher KCl values, at the depth of this depression, the P.D. change is *greatly diminished*, instead of being merely displaced downward around a new and lower position, which would be expected

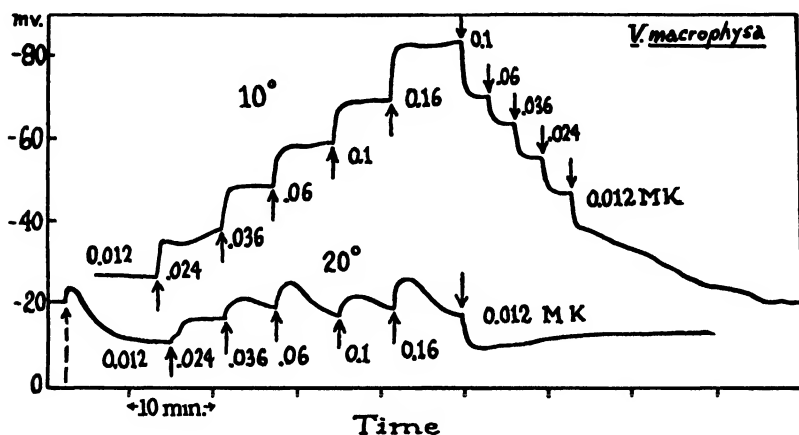


FIG. 7. An extreme case of the influence of temperature upon the potassium effect (*V. macrophysa* in this case). While good stepwise increments were obtained with the increases of KCl shown at 10° (upper curve), only temporary cusps were given by the same cell when similarly exposed at 20° (lower curve), the P.D. falling after the cusps to an almost constant base line. At 30–35° more rapid recovery tends to ensue after these cusps in such a series. P.D. in millivolts; time marks 10 minutes apart. Note drop on warming at first (dotted) arrow of lower curve.

in scheme (a). There is a real loss, partial or complete, of the potassium effect during the trough; it recovers rapidly if sea water is restored, or more slowly if the KCl remains. The cause of this "alteration" by potassium itself is not clear, but it is conditioned among other things by the pH of the sea water, being almost entirely abolished at high pH.<sup>9</sup> Clearly, temperature also conditions it, for there is no trough, and no corresponding loss of potassium effect at 15°; while it becomes prominent at 25°. The situation at 35° is then interesting: what causes the very slight trough and final very large potassium effect? Apparently the higher temperature greatly speeds up the spontaneous recovery from the alteration, which occurs more slowly and incompletely at 25°. This may well be some metabolic response to the altered pH due to the entrance of KOH; it may even cause the curve after recovery to

rise higher than at 15°. Fig. 7 shows a striking example of these alterations at 20°, which were prevented at 10°. The picture for pH is very similar to this and will be reported elsewhere.

(c) A third possibility involves the accumulatory mechanism, which carries the entering potassium over across *Y* into the vacuole. This is clearly conditioned by metabolism, and might therefore be expected to have a high temperature coefficient. We may assume that (as in *a*) little KCl moves across *X* at 15°, so that the original gradient is maintained, hence a high P.D. At 25°, however, considerably more KCl enters *X*, but is not very rapidly moved out of the protoplasm across *Y* to the sap by the accumulatory mechanism. At 35°, finally, despite a large entrance across *X*, the accumulatory mechanism is very active, and keeps the protoplasm well depleted of the entering potassium, hence the P.D. is high. There is evidence that in *Nitella* radioactive potassium does enter the protoplasm readily (across *X*), but only slowly accumulates in the sap.<sup>13</sup> The entrance is also rhythmic, somewhat like the later rises and falls of the KCl time curves in *Valonia*. The behavior of radioactive potassium in the protoplasm of *Valonia* at different temperatures would be interesting to study in connection with the bioelectric curves.

Choice between (*a*), (*b*), and (*c*) is difficult, but the author inclines toward (*b*) ("alteration") as having the most experimental proof at the moment. By permitting a temporary approach or equality of mobility for  $K^+$  and  $Cl^-$ , this could govern the effects described, although the other possibilities (*a*) and (*c*) cannot yet be dismissed.

It is evident that (*b*) might account for the temperature effects in ordinary sea water since the P.D. in the steady state after "recovery" would vary in the manner observed. In (*a*), however, any change in the required direction might be only temporary and the steady state after "recovery" would not be predictable without additional assumptions. In (*a*) and (*c*) it would be necessary to assume that in ordinary sea water there is enough inward movement of  $K^+$ , due to growth, to account for the observed effects.

These considerations emphasize again the remarkable lability of the protoplasmic surface, which Osterhout has demonstrated with a variety of chemical agents in *Valonia* and other plant cells.<sup>14</sup> Temperature, in influencing the alteration of the surface by potassium itself, is therefore but another agent in producing this "Osterhout effect."<sup>15</sup>

<sup>13</sup> Brooks, S. C., *J. Cell. and Comp. Physiol.*, 1939, **14**, 383.

<sup>14</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1936-37, **20**, 13, 685; 1940-41, **24**, 311, 699; *J. Cell. and Comp. Physiol.*, 1941, **18**, 129.

<sup>15</sup> The large positive P.D. of *Halicystis*, which has its probable origin in the organic salts of the protoplasm,<sup>11</sup> is only slightly influenced by temperature.<sup>5</sup> If, however, the KCl content of the sea water is increased, the temperature effects are considerably enhanced.<sup>11</sup> The mechanism is probably that of (*b*) described above, and will be discussed at length in another paper.

## SUMMARY

The effect of temperature upon the bioelectric potential across the protoplasm of impaled *Valonia* cells is described. Over the ordinary tolerated range, the P.D. is lowest around 25°C., rising both toward 15° and 35°. The time curves are characteristic also. The magnitude of the temperature effect can be controlled by changing the KCl content of the sea water (normally 0.012 M): the magnitude is greatly reduced at 0.006 M KCl, enhanced at 0.024 M, and greatly exaggerated at 0.1 M KCl.

Conversely, temperature controls the magnitude of the potassium effect, which is smallest at 25°, with a cusped time course. It is increased, with a smoothly rising course, at 15°, and considerably enhanced, with only a small cusp, at 35°. A temporary "alteration" of the protoplasmic surface by the potassium is suggested to account for the time courses. This alteration does not occur at 15°; the protoplasm recovers only slowly and incompletely at 25°, but rapidly at 35°, in such fashion as to make the P.D. more negative than at 15°. This would account for the temperature effects observed in ordinary sea water.

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